Effects of Ocean Acidification on the nutritional quality of Antarctic phytoplankton as food for *Euphausia superba* larvae

by

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The research associated with this thesis abides by the international and Australian codes on human and animal experimentation, the guidelines by the Australian Government's Office of the Gene Technology Regulator and the rulings of the Safety, Ethics and Institutional Biosafety Committees of the University.

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Martin Luther King, Jr: We are now faced with the fact that tomorrow is today. We are confronted with the fierce urgency of now. In this unfolding conundrum of life and history there is such a thing as being too late… We may cry out desperately for time to pause in her passage, but time is deaf to every plea and rushes on. Over the bleached bones and jumbled residue of numerous civilizations are written the pathetic words: "Too late."

Abstract

As a consequence of human activity carbon dioxide (CO_2) concentration has risen from ~280 parts per million (ppm) pre-industrial to ~397ppm today. About 30% of anthropogenic CO_2 emissions have been taken up by the oceans, raising the seawater partial pressure of CO_2 (pCO_2) while lowering seawater pH. This change in carbonate chemistry has the potential to alter phytoplankton biochemistry, physiology and species composition, thereby affecting the quality and quantity of the basis of the food web. Little is known about how this may flow on to affect dependent grazers, particularly in polar regions. In the Southern Ocean, Antarctic krill, *Euphausia superba,* is the key species that supports many predators. Negative impacts on the abundance and quality of this species could have far reaching consequences for the Antarctic food web. To address this knowledge gap, the aim for this research project was to assess the potential of ocean acidification to affect Antarctic krill through impacts on Antarctic phytoplankton as their food.

To investigate the effects of ocean acidification on phytoplankton as food for krill, a culture system was designed and developed that could maintain phytoplankton growing exponentially for extended periods at different pCO_2. The phytoplankton could then be fed to krill over a time period sufficient to detect physiological changes in the krill. As a first step, a semi-continuous culture system was developed and used. Furthermore, only phytoplankton

cultures were exposed to elevated pCO_2, while krill larvae were maintained at ambient pCO_2 in order to ascertain the direct effects of feeding on phytoplankton grown at elevated pCO_2. A pilot study using the diatom *Synedropsis hyperborea* did not reveal strong CO_2 perturbations in either the alga or the krill feeding on it. A follow-up experiment with the ubiquitous diatom *Pseudo-nitzschia subcurvata* cultured at elevated pCO_2 showed subtle changes which included a decrease in the nutritionally important long-chain ($>C_{20}$) polyunsaturated fatty acids (PUFA) (22:6ω3, DHA and 20:5ω3, EPA) and a small increase in cellular carbohydrate concentrations. Krill larvae fed *Pseudo-nitzschia subcurvata* cells grown at 896µatm CO_2 had significantly higher mortality rates than those larvae fed algae grown at ambient CO_2 concentrations and this was attributed to the CO_2-induced changes in the nutritional quality of *Pseudo-nitzschia subcurvata* cultured at elevated pCO_2.

During the course of early experiments, a number of limitations and scope for improvements of the semi-continuous culture system were identified and therefore a continuous culture method was developed. This system greatly reduced the maintenance time for phytoplankton cultures, and reduced variation in experimental pCO_2 treatments over time. With this new culture system three more Antarctic phytoplankton species, *Pyramimonas gelidicola*, *Phaeocystis antarctica* and *Gymnodinium sp.* were assessed for their susceptibility to elevated pCO_2. The observed changes were species-specific, variable and often subtle, and included changes in the fatty acid composition and cellular carbohydrate concentration.

Exposing high CO_2 grown phytoplankton cells for up to two days to ambient pCO_2 seawater before being replaced by fresh culture was a limitation of the previously used semi-continuous culture system. A final experiment therefore aimed to establish whether exposing phytoplankton for two days to a different pCO_2 environment altered the algal biochemistry and had thus potentially compromised the previous experimental design. I cautiously conclude that a rapid change in CO_2 concentration did not affect the biochemistry of *Pseudo-nitzschia subcurvata*, however, this may be attributed to an unexpected depletion of nutrients during the experiment.

In conclusion, the phytoplankton response to elevated CO_2 concentrations was species-specific and mostly subtle. Based on the final experiment, it seems that the impacts of CO_2 concentrations could be small when compared to the effects of other abiotic factors such as nutrient concentration. Future phytoplankton research should therefore focus on elucidating the combined effects of elevated CO_2 concentrations and concurrent changes in environmental parameters such as, seawater temperature, light intensities and / or nutrient concentration as mediated by global climate change. The increase in krill mortality when feeding on high CO_2 grown *Pseudo-nitzschia subcurvata* cells confirmed that ocean acidification can negatively impact Antarctic krill indirectly through its food source. However, to be able to extrapolate these results from the laboratory to nature, more Antarctic phytoplankton species must be tested and the combined effects of altered carbonate chemistry and food quality need to be further investigated. In addition to the changes occurring to individual phytoplankton species,

changes to the community composition need to be understood as well as the possibility of selective feeding by krill which might allow them to compensate for possible changes in the quantity and quality of their food source.

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List of Abbreviations

ANOVA = analysis of variance

C = carbon

Ca^{2+} = calcium ion

$CaCO_3$ = calcium carbonate

CCM = carbon concentrating mechanism

CHO = carbohydrate

Chl a = Chlorophyll a

CO_2 = carbon dioxide

CO_3^{2-} = carbonate ion

Ddx = diadinoxanthin

DHA = docosahexaenoic acid, 22:6ω3

DIC = dissolved inorganic carbon

dtx = diatoxanthin

EPA = eicosapentaenoic acid, 20:5ω3

FAME = fatty acid methyl esters

GAM = generalized additive model

GC = gas chromatography

Gt = giga ton = 10^{15} g

H^+ = proton

H_2CO_3 = carbonic acid

HCl = hydrochloric acid

HCO_3^- = bicarbonate ion

IPCC = Intergovernmental Panel on Climate Change

K'_{sp} = apparent solubility product

µatm = micro atmospheres (unit of CO_2 concentration in seawater)

MUFA = monounsaturated fatty acids

N = nitrogen

$NaHCO_3$ = sodium bicarbonate

NaOH = sodium hydroxide

O_2 = oxygen

P = phosphorus

pCO_2 = partial pressure of CO_2

pH = logarithmic scale of hydrogen ions

PL = polar lipids

ppm = parts per million (unit of CO_2 concentration in the atmosphere)

PUFA = polyunsaturated fatty acids

RubisCO = ribulose-1,5-bisphosphate carboxylase / oxygenase

SFA = saturated fatty acids

TAG = triacylglycerol

TFA = total fatty acids

TLC – FID = flame ionization detector

Tris (buffer) = tris-(hydroxymethyl)-aminomethane

UV = ultra violet

Ω = calcium carbonate saturation state

WE = wax ester

1. General Introduction

1.1. The carbon cycle

Carbon (C) is part of all major building blocks in living creatures. Primary producers transform it from inorganic, such as carbon dioxide (CO_2), to organic molecules such as carbohydrates and lipids. Next to the cycling of water, the carbon cycle is central to Earth's climate system and C is continuously cycling through various reservoirs at their associated timescales. The biggest C reservoirs on Earth are marine sediments (30million Gt = 10^{15} g), ocean (38,000Gt), terrestrial biosphere (2000Gt), fossil fuels and shales (12,000Gt), and the atmosphere (700Gt) (Fig. 1.1) (The Royal Society 2005). Over geologically short time scales such as 10 – 100 years, C flux to the atmosphere from biological respiration and outflow through photosynthesis represent the biggest fluxes between C reservoirs. Changes in the atmospheric CO_2 concentration, such as seen in the glacial-interglacial cycles, are on the timescale of hundreds of thousands of years and are due to the equilibration between atmosphere and ocean. On an even larger timeframe, such as millions of years and more, atmospheric CO_2 concentration is influenced by rock weathering, subduction of C sediments and releases from volcanoes (Hethertington and Raven 2005).

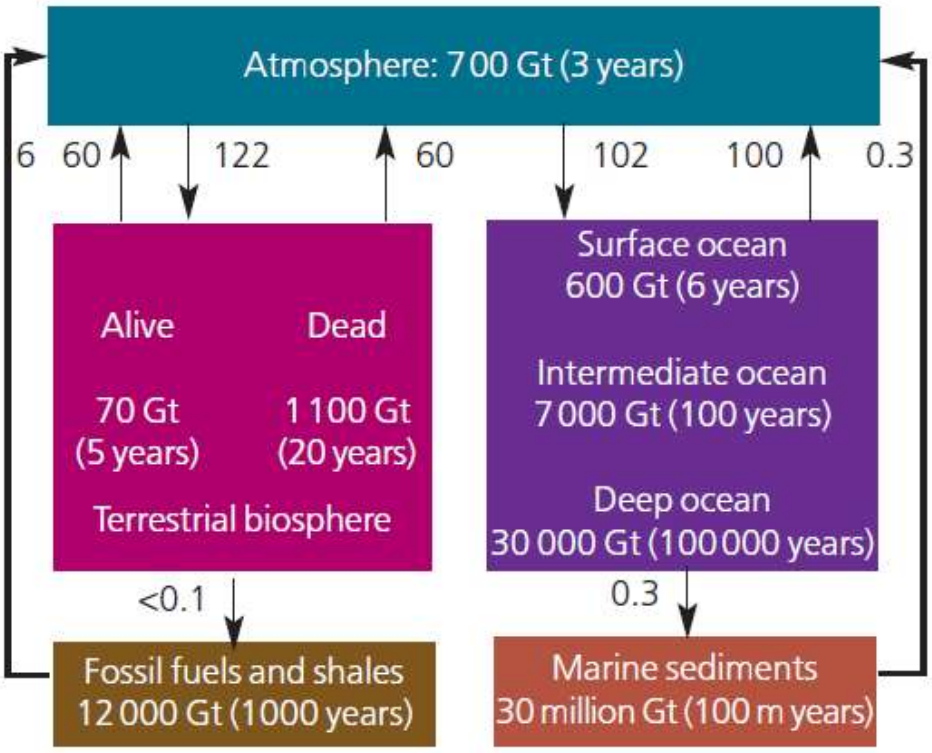

Figure 1.1 The global carbon cycle, including contributions from human activity. Reservoir sizes are in Gt of carbon (= 10^{15} g) and fluxes between them are in Gt per year. Associated residence times are in brackets (The Royal Society 2005).

Although the uptake of CO_2 per unit area in the ocean is smaller than that of most terrestrial ecosystems, due to the vast expanse of the ocean on Earth, the total uptake of CO_2 by photosynthetic organisms in the ocean is about equal to that of terrestrial photosynthetic species (Field *et al.* 1998; The Royal Society 2005). The uptake of inorganic C by autotrophic organisms in the ocean is called the "biological pump". According to models, atmospheric CO_2 would almost double if all biological production in the ocean stopped (Maier-Reimer *et al.* 1996). The second important ocean C sink is the physical pump or solubility pump, resulting from elevated solubility of CO_2 in cold, down-welling waters (Wolf-Gladrow *et al.* 1999). On land and in the ocean the majority of C is recycled within the biosphere, i.e. food webs and microbial

loops. A small proportion is, however, deposited in the soil or sediment forming fossil fuel and other deposits. Changes to the rate of fluxes and / or the release of C deposits have been shown to drive major changes in climate, such as the glacial-interglacial cycles of the past (Petit *et al.* 1999; Zachos *et al.* 2005; Nisbet *et al.* 2009; DeConto *et al.* 2012; Shakun *et al.* 2012). Since the industrial revolution humans are altering the amount of C in circulation and the rate of C fluxes by burning fossil fuel deposits, cement production and by making changes in terrestrial vegetation with subsequent changes in the world's climate.

1.2. Climate change

"First, I worry about climate change. It's the only thing that I believe has the power to fundamentally end the march of civilization as we know it, and make a lot of the other efforts that we're making irrelevant and impossible." (Bill Clinton)

Global climate change is the unintentional result of the industrial revolution with unforeseeable consequences for future generations and ecosystems. Since the beginning of the industrial revolution about 345Gt carbon have been released into the atmosphere through human activities, including oil and coal based energy production, deforestation and land use change (Tans 2009). Carbon dioxide concentration has risen from about 280 parts per million by volume (ppm) during pre-industrial times to about 397ppm today (ProOxygen 2007-2011; Blasing 2013), a level not seen for the last 650,000 years and probably even the last 20 million years (Berner 1990; Hoenisch *et al.* 2009).

Half of this increase has occurred over the last 30 years (Feely *et al.* 2009). Today's rate of increase is 100 times faster than occurred ever before during the previous 650,000 years and 10 times faster than 55 million years ago, when seawater pH was as low as expected by the end of this century (The Royal Society 2005; Schiermeier 2011) (Fig. 1.2).

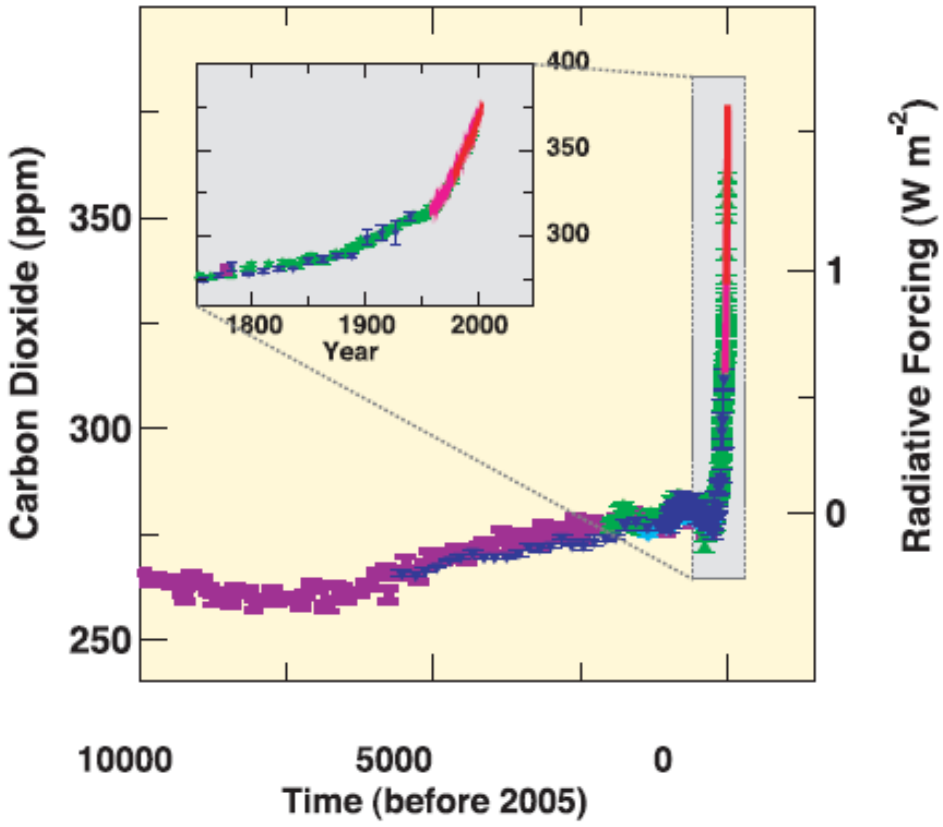

Figure 1.2 Increase in atmospheric carbon dioxide concentration over the last 10,000 years (Bernstein *et al.* 2007).

Anthropogenically released long-lived greenhouse gasses, such as CO_2, methane, nitrous oxide, halocarbons and sulphur hexafluoride, accumulate in the atmosphere and increasingly absorb and radiate back heat from the Earth's surface and lower atmosphere. This phenomenon is called the human induced "greenhouse effect" and causes average global temperature to rise (The Royal Society 2010). Between 1906 and 2005 global surface temperature increased

by 0.74 degrees (Hegerl *et al.* 2007) and is projected to increase by 0.3°C to 0.7°C between 2016 – 2035 relative to 1986 – 2005 temperatures (IPCC 2013). Comprising over 70% of the surface of the Earth, the oceans have the capacity to store more than 1000 times the heat compared to the atmosphere (Levitus *et al.* 2005). Therefore, 90% of the additional heat in the earth system has been stored in the ocean since the 1960s (Bindoff *et al.* 2007), resulting in an increase of average ocean temperature by 0.1°C in the upper 700m (Wohlers *et al.* 2009).

A consequence of global warming is global sea level rise. Global sea levels have risen approximately 3.1mm/yr between 1993 and 2003, due to thermal expansion and the melting of glaciers and ice caps. Snow cover on land and sea ice is retreating and precipitation patterns are changing (Bernstein *et al.* 2007) (Fig. 1.3). Global mean sea level is projected to rise at least between 0.17 and 0.32m for the period of 2046 – 2065 relative to the 1986 – 2005 period (IPCC 2013).

Together these human induced changes, amongst others, to our physical environment and climate are referred to as “climate change”.

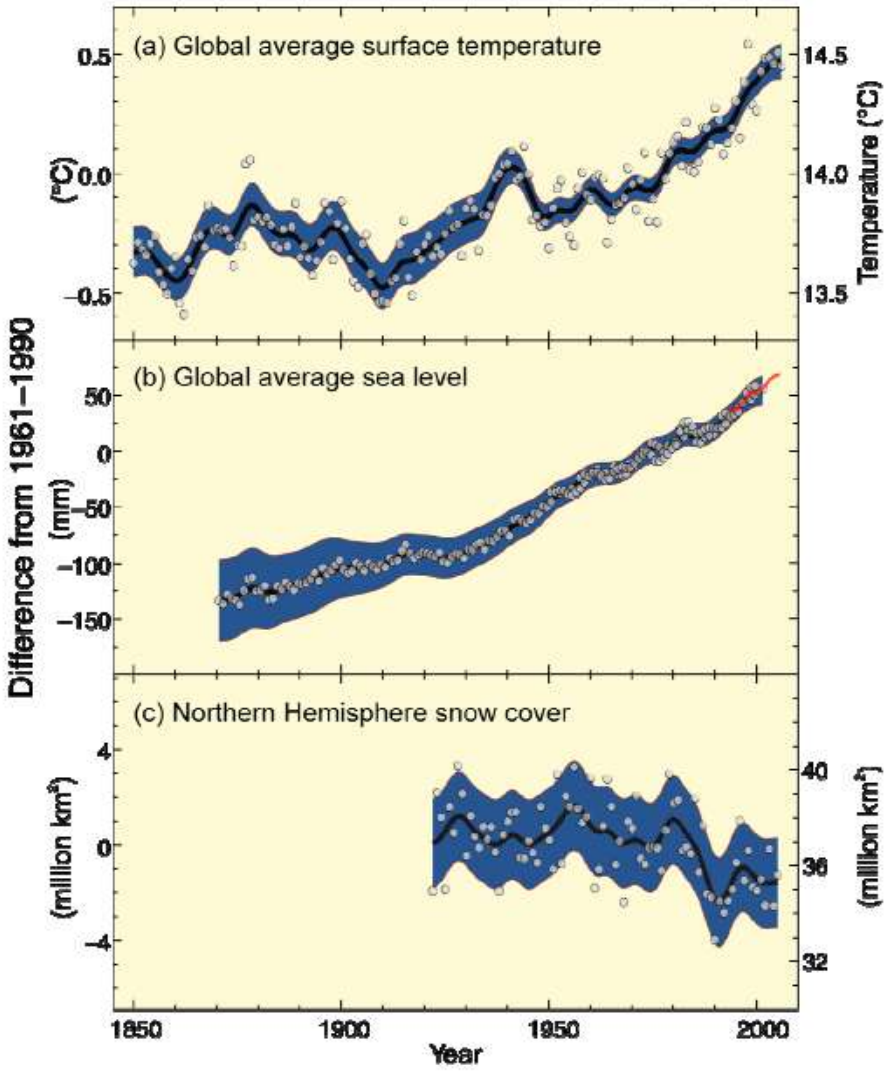

Figure 1.3 Change in global average surface temperature, sea level and Northern Hemisphere snow cover between 1961 and 1990 due to climate change (Bernstein *et al.* 2007).

1.3. Ocean acidification

The ocean and atmosphere are in equilibrium in terms of CO_2 concentration. About 30% of anthropogenic CO_2 emissions have been taken up by the oceans. This continuous absorption of CO_2 is changing the oceans chemical properties (Sabine *et al.* 2004) and is a process termed 'ocean acidification'. CO_2 reacts with seawater, forming bicarbonate ions (HCO_3^-) and protons (H^+) thus reducing pH. The excess protons react with carbonate ions (CO_3^{2-}) to produce

more HCO_3^- ions. This process results in a reduction in the concentration of CO_3^{2-} and is thereby lowering the calcium carbonate ($CaCO_3$) saturation state (Ω) (Wolf-Gladrow *et al.* 2007) (Fig. 1.4). Total alkalinity, the excess of proton acceptors over proton donors does not change with the absorption of CO_2 (Wolf-Gladrow *et al.* 2007).

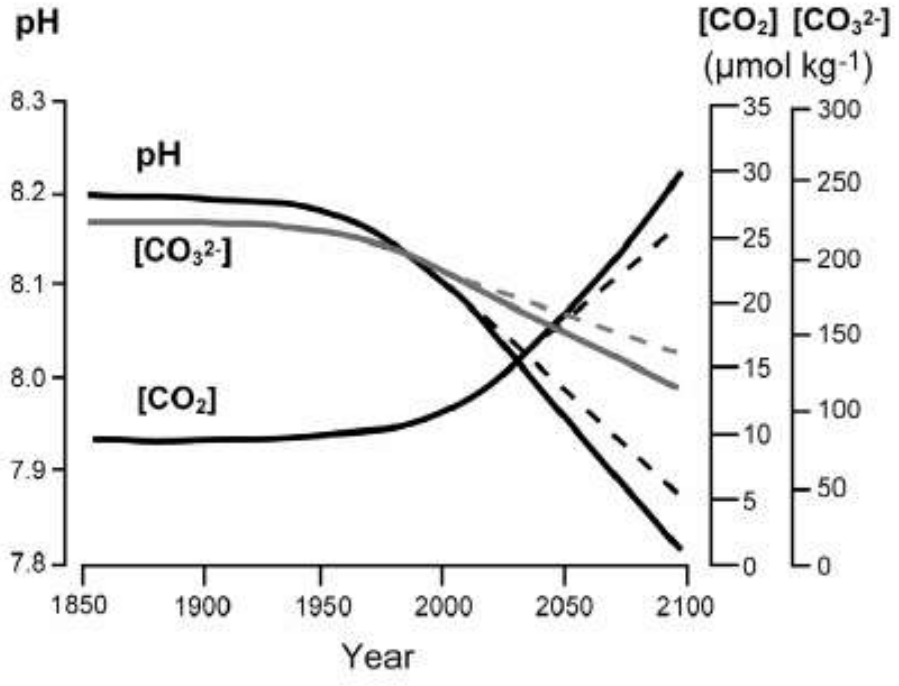

Figure 1.4 Seawater pH, dissolved carbon dioxide (CO_2) and carbonate ion (CO_3^{2-}) concentration in the surface layer under the "business as usual" CO_2 emission scenario (IS92a) (Houghton *et al.* 1995). Dashed lines represent predicted changes under the Kyoto protocol CO_2 emission scenario (modified after Wolf-Gladrow *et al.* 1999).

Since pre-industrial times, average global surface ocean pH has dropped by 0.1 units, which equates to a 30% increase in H^+ concentration (Caldeira and Wickett 2003; The Royal Society 2005). Under current CO_2 emission rates, pH will decrease by 0.5 units by 2100 (The Royal Society 2005) and, under the assumption that atmospheric CO_2 concentration will peak at

approximately 1900ppm, pH could drop by 0.77 units by 2300 (Caldeira and Wickett 2003) (Fig. 1.5).

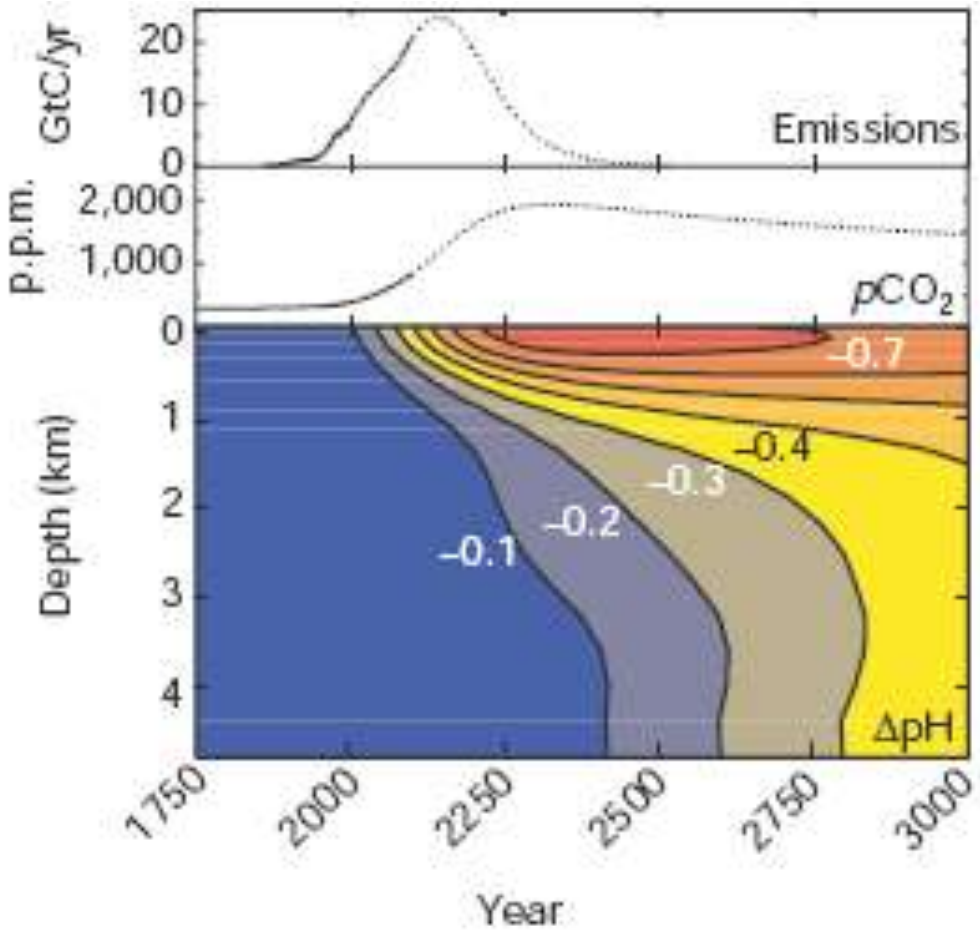

Figure 1.5 Average global ocean pH and its depth distribution in relation to CO_2 emissions in Gt C/yr and atmospheric CO_2 concentration in ppm (Caldeira and Wickett 2003).

Acidification of the oceans is not occurring at the same pace across the planet. High latitude oceans are particularly vulnerable for a number of reasons. More than 40% of anthropogenic CO_2 in the ocean enters via the Southern Ocean (Khatiwala *et al.* 2009). Due to lower seawater temperatures, in which more CO_2 can dissolve, high latitude oceans have the capacity to physically take up more CO_2 than low latitude oceans. Furthermore, the ocean acidification – induced changes are occurring on top of a seasonally highly variable

background seawater pCO_2. Deep water formation brings CO_2 into the deep ocean, away from direct contact with the atmosphere. Upwelling and subsequent entrainment of CO_2-rich deep waters during winter causes the reverse (McNeil and Matear 2008; Fabry *et al.* 2009). Intense phytoplankton blooms in summer and limited primary productivity in winter due to light limitation together with wind mixing and deep water upwelling, make the Antarctic Ocean one of the most seasonally variable in regards to surface ocean pCO_2 and associated carbonate chemistry parameters, such $CaCO_3$ saturation state (McNeil *et al.* 2011). Although little is known about the effects of ocean acidification on polar organisms (Fabry *et al.* 2009) and particularly Antarctic phytoplankton (Riebesell 2004; Montes-Hugo *et al.* 2009), there are indications that the effects are mostly negative and that species occupying high latitudes will be among the first to be affected by ocean acidification (Andersson *et al.* 2008; Fabry *et al.* 2009). Marine primary producers absorb up to half of global atmospheric CO_2 (Wolf-Gladrow *et al.* 1999; Falkowski and Raven 2007; Finkel *et al.* 2010). Any changes in the efficiency of this process will have feedback effects on the global carbon cycle and thus global climate.

1.4. Impacts of ocean acidification on marine organisms

1.4.1. Direct effects of ocean acidification

Ocean acidification has the potential to affect marine organisms directly through increased availability of CO_2 (Hein and Sand-Jensen 1997; Burkhardt *et al.* 2001; Engel *et al.* 2005; Poertner and Farrell 2008), increased acidity

(Doney 2006; Fabry *et al.* 2010; Gaylord *et al.* 2011), reduced calcium carbonate saturation state (Doney 2006) and altered bioavailability of trace metals (Millero *et al.* 2009).

Early research mostly concentrated on calcifying taxa such as corals and calcifying plankton, e.g. coccolithophorids, pteropods and foraminifera (Spero *et al.* 1997; Bijma *et al.* 1999; Kleypas *et al.* 1999; Marubini and Atkinson 1999; Riebesell *et al.* 2000), due to the concern over calcium carbonate dissolution in acidified and CO_3^{2-} poorer waters. Calcifying organisms have either internal or external calcium carbonate shells and there are four forms of calcium carbonate: high-magnesium calcite, aragonite, low-magnesium calcite and calcite, listed in order of increasing stability (Andersson *et al.* 2008). Major pelagic producers of calcite and aragonite are coccolithophores and pteropods, respectively. The most common high-magnesium calcite producers are red coralline algae, benthic foraminifera, bryozoans and echinoderms and to varying degrees also crustaceans and molluscs (Jin *et al.* 2006; Andersson *et al.* 2008 and references therein; Hunt *et al.* 2008). Each of these mineral forms has a saturation state Ω dependent on the concentration of Ca^{2+} and CO_3^{2-} ions in the water and a constant K'_{sp}, the apparent solubility product. This constant is specific to each calcium carbonate mineral form and varies with temperature, salinity and pressure (Feely *et al.* 2009).

$$\Omega = [Ca^{2+}] * [CO_3^{2-}] / K'_{sp}$$

At Ω equal to 1, calcium carbonate is naturally precipitated at the same rate as it is dissolved, and at Ω below 1 calcium carbonate dissolves faster than it precipitates (Atkinson and Cuet 2008).

Marine calcifying organisms can excrete calcium carbonate exoskeletons and shells in waters with Ω greater than 1, however, most species depend on Ω to be greater than 3. Coral reef growth and development has been shown to cease around 3.0 to 3.3 (Atkinson and Cuet 2008 and ref therein) and today's tropical surface seawater aragonite saturation state is predicted to decrease from 3.0 - 3.5 to 2.0 - 2.5 in the next 100 years (Orr *et al.* 2005). Models predict surface waters in the Southern Ocean to be under-saturated with respect to aragonite by 2050 under the IPCC 'business-as-usual' scenario IS92a (Orr *et al.* 2005). Taking winter seasonal upwelling of CO_3^{2-} poor deep waters in the Southern Ocean into account, McNeil and Matear (2008) predict aragonite under-saturation as early as 2030. Even before the oceans become under-saturated with respect to calcium carbonate, calcifiers are likely to be negatively affected (Doney *et al.* 2009). The closer the seawater calcium carbonate saturation state moves to 1 the more energy intensive it is for calcifying organisms to precipitate calcium carbonate and maintain their exoskeleton (Fabry *et al.* 2008; Pelejero *et al.* 2010). This sub-lethal effect could pose a fitness disadvantage for species in competition with others and therefore lead to species regime shifts (The Royal Society 2005; Veron *et al.* 2009).

Although increased CO_2 concentration in the ocean acidifies the seawater, elevated availability of CO_2 could be advantageous to photosynthesising organisms (Egge *et al.* 2009). Phototrophic microorganisms, such as marine phytoplankton, use the enzyme ribulose-1,5-bisphosphate carboxylase / oxygenase (RubisCO) to fix CO_2. RubisCO has a half saturation constant for CO_2 that is much higher than present CO_2 concentrations, which means that it has a low affinity for CO_2 at ambient pCO_2 (Badger *et al.* 1998). Therefore some algae have developed carbon concentrating mechanisms (CCMs) to increase the CO_2 concentration around RubisCO and thus its carboxylating performance. Since these CCMs are believed to be energetically costly (Hethertington and Raven 2005) an increase in the surrounding CO_2 concentration would allow a down-regulation of CCMs and thereby provide the species with an energetic advantage over species that do not possess CCMs. This theory is supported by the finding that certain phytoplankton species exhibit increased growth rates under elevated pCO_2 (Clark and Flynn 2000; Beardall and Raven 2004; Wu *et al.* 2010; McCarthy *et al.* 2012), whereas other studies found no or only small effects of elevated CO_2 levels on growth rates of other species (Burkhardt *et al.* 1999; Boelen *et al.* 2011).

While reduced pH and altered seawater carbonate chemistry are the most apparent threats of ocean acidification, trace metal bioavailability may also be affected. Decreasing seawater pH will impact on the thermodynamics and kinetics of metals, such as iron, causing changes in their solubility and adsorption and therefore availability and toxicity to marine organisms (The Royal Society 2005; Barry *et al.* 2008; Millero *et al.* 2009). A drop in

seawater pH increases the proportion of soluble iron available for phytoplankton (Raven 1986), however, it also makes toxic forms of metals biologically more available as free dissolved forms rather than occurring as complex forms (The Royal Society 2005).

It should be mentioned here that the above outlined direct effects of ocean acidification only consider elevated pCO_2 and the associated changes in carbonate chemistry. However, ocean acidification will not impact ecosystems in isolation (Byrne and Przeslawski 2013). In the future high – CO_2 world temperatures will also be higher. This will cause an increase in ocean surface stratification and a decrease in upwelling of nutrient rich deep waters, while alleviating light limitation by mixing in some areas and intensifying UV-radiation exposure in others (Hoegh-Guldberg and Bruno 2010; Boyd 2011). These factors are, however, beyond the scope of this research project.

1.4.2. Indirect effects of ocean acidification through impacts on the base of the food web

In every food web, energy enters through autotrophic species called primary producers at the bottom trophic level; grazers feed on these organisms and are in turn eaten by predators. Removal of any such primary producing species, reduction in their abundance and / or deterioration in their nutritional quality can have major ramifications for the entire food web. Shifts in the timing of phytoplankton blooms and the development of grazer larvae have equally been identified as potential adverse consequences of climate change (Sommer *et al.* 2007; Hauri *et al.* 2009).

It is well known that some phytoplankton species are superior food for grazers due to their favourable fatty acid profiles (Nichols 2003; Arendt *et al.* 2005; Chen *et al.* 2012). Diatoms are often referred to as inadequate or even toxic food for grazers. Jones and Flynn (2005) have shown that single diatom species on their own are nutritionally insufficient as a food source for the copepod *Acartia tonsa*, while normal growth is supported by a mixed diatom species diet. Since marine organisms have varying capabilities to cope with changing conditions, phytoplankton species composition could be altered by ocean acidification (Hein and Sand-Jensen 1997; Tortell *et al.* 2002; Hays *et al.* 2005; The Royal Society 2005; Riebesell *et al.* 2007) and thereby the prevalence and proportions of nutritionally important compounds such as key or essential polyunsaturated fatty acids and the ratio of protein to carbohydrate. Elevated CO_2 concentration has been shown to influence the species composition of communities and ratios of carbon to nutrient uptake rates (Sambrotto *et al.* 1993; Burkhardt and Riebesell 1997; Riebesell *et al.* 2007; Bellerby *et al.* 2008; Paulino *et al.* 2008). This could lead to changes in C:N:P ratios and nutritional quality of phytoplankton (Thingstad *et al.* 2008; Fiorini *et al.* 2010). An ocean acidification induced change in the species composition of natural phytoplankton communities could therefore alter the overall nutritional quality of phytoplankton communities for grazers.

Species composition is one defining factor in the overall nutritional quality of phytoplankton, however, equally important factors are the elemental composition and biochemistry of individual species. The physiology and elemental composition of phytoplankton are directly affected by

environmental factors such as light intensity and nutrient concentrations (Shifrin and Chisholm 1981; Mayzaud *et al.* 1990; Arts *et al.* 1997; Burkhardt and Riebesell 1997 and references therein). CO_2 concentration and pH have also been shown to affect the biochemistry of phytoplankton, such as a decrease (~20% at pH = 7.9 Thornton 2009) or increase (~23% at pH = 7.5 Taraldsvik and Myklestad 2000) in cellular carbohydrate concentrations, an increase in cellular carbon to nitrogen ratio (~70% at 800µatm CO_2 Schoo *et al.* 2013) and a decrease in the concentration of polyunsaturated fatty acids (~36% at 761µatm CO_2 Rossoll *et al.* 2012), with some species showing no change in some biochemical parameters to elevated pCO_2 (Table 1). It has been shown that the biochemistry of phytoplankton and changes therein can affect grazer growth and reproduction (Rothhaupt 1995; Urabe *et al.* 2003; Chen *et al.* 2012; Rossoll *et al.* 2012; Schoo *et al.* 2013). The composition of fatty acids, most importantly polyunsaturated fatty acids (PUFA), are critically important for grazer development, reproduction and hatch rates, as has been well researched by the aquaculture industry amongst others (Koven *et al.* 1989; Bell *et al.* 2007; Yoshida *et al.* 2011; Chen *et al.* 2012). Altering the composition of phytoplankton communities, as well as their availability and nutritional quality, could therefore have major ramifications for higher trophic levels, trophodynamics and ecosystem functioning (Urabe *et al.* 2003; Carotenuto *et al.* 2007; Hauri *et al.* 2009; Montes-Hugo *et al.* 2009).

Table 1 Literature review of biochemical response to above ambient pCO_2 in phytoplankton. C:N = carbon to nitrogen ratio, CHO = cellular carbohydrate content, PUFA = polyunsaturated fatty acid content

Species		Biochemical parameter	Qualitative response to elevated pCO_2	Reference
Skeletonema costatum	Diatom	C:N	No significant change	(Burkhardt and Riebesell 1997)
Phaeodactylum tricornutum	Diatom	C:N	No significant change	(Burkhardt *et al.* 1999)
Skeletonema costatum	Diatom	C:N	No significant change	
Asterionella glacialis	Diatom	C:N	No significant change	
Thalassiosira weissflogii	Diatom	C:N	No significant change	
Thalassiosira punctigera	Diatom	C:N	No significant change	
Coscinodiscus wailesii	Diatom	C:N	No significant change	
Scrippsiella trochoidea	Dinoflagellate	C:N	No significant change	
Rhodomonas salina	Cryptophyte	C:N	Increase	(Schoo *et al.* 2013)
Proboscia alata	Diatom	C:N	Increase	(Hoogstraten and Timmermans 2012)
Chaetoceros muelleri	Diatom	CHO	Decrease	(Thornton 2009)
Skeletonema costatum	Diatom	CHO	Increase	(Taraldsvik and Myklestad 2000)
Thalassiosira pseudonana	Diatom	PUFA	Decrease	(Rossoll *et al.* 2012)
Nitzschia lecointei	Diatom	PUFA	Decrease only at -1.8°C No difference at +2.5°C	(Torstensson *et al.* 2013)

1.5. Antarctic krill – a key species in the Antarctic food web

Food webs are complex systems, often connecting a large number of species through the transfer of energy from the bottom to the top. Food webs are rarely linear and predators usually rely on a number of prey species. Key species form a central part of food webs. In the Southern Ocean food web Antarctic krill, *Euphausia superba*, is a key species. Krill forms a vital part in the diet of many animals in the Southern Ocean such as seals, seabirds, penguins and whales (Atkinson *et al.* 2004). Apart from sustaining higher trophic levels, and being a target for fisheries itself, krill products are also of commercial interest for humans and in animal nutrition (Virtue *et al.* 1993; Martin *et al.* 2006; Kawaguchi and Nicol 2007; Simmons and Isaac 2007).

1.5.1. Possible direct and indirect effects of ocean acidification on Antarctic krill

Antarctic krill could be susceptible to direct and indirect effects of climate change in a number of ways due to their life history traits and habitat. Yet little is known about the vulnerabilities of this species to the effects of ocean acidification (Kawaguchi *et al.* 2011).

Like all crustaceans, krill undergo a process called moulting. Krill shed their exoskeleton at regular intervals throughout their life in order to grow, or shrink in times of low food supply (Dixon and Ikeda 1982b; Dixon and Ikeda 1982a). The exoskeleton is a chitinous membrane supported by calcium carbonate ($CaCO_3$), however, compared to other decapods Antarctic krill has

been regarded as a poorly calcified species with relatively low calcium levels in the integument and whole body (Nicol *et al.* 1992). Currently it is unknown whether krill possess the more stable calcite or more soluble aragonite form of $CaCO_3$, yet in both cases it is possible that lower seawater pH and $CaCO_3$ saturation state could affect calcification rates and post-moult calcification (Whiteley 2011).

An increase in seawater acidity could also negatively affect krill metabolic rate, growth and reproduction (Whiteley 2011), depending on the capability of krill for iono- and osmo – regulation. Recent experiments showed that krill embryos developed normally under pCO_2 of up to 1000µatm, with almost complete developmental inhibition at 2000µatm CO_2 (Kawaguchi *et al.* 2011). Hatch rates of eggs exposed to 1500µatm were approximately 40% of eggs reared at 380µatm (Kawaguchi *et al.* 2013). CO_2 concentrations in deep waters are naturally higher than at the surface, primarily due to circulation and respiration processes along with colder temperatures and higher pressures (Feely *et al.* 2009). Since krill migrate between the surface and great depth (Schmidt *et al.* 2011), they already encounter pCO_2 of up to 600µatm (Kawaguchi *et al.* 2011). This suggests that they have effective mechanisms to maintain acid-base and osmo – homeostasis and are well adapted to moderate changes in seawater pH. CO_2 concentrations at depth are, however, predicted to increase to around 1400µatm within a century in some regions of the Southern Ocean under the 'business-as-usual' emissions scenario (Kawaguchi *et al.* 2011) and therefore krill will be exposed to much lower seawater pH

than surface dwelling animals as krill vertically migrate through the water column.

Krill larval development includes life history traits that make them particularly susceptible to indirect effects of ocean acidification, such as deterioration in phytoplankton nutritional quality. Females spawn in surface layers in early austral summer, generally offshore where waters are deeper (Siegel 1988). The eggs sink to a few hundred to a thousand metres or more (Nicol 2006). During their descent, the eggs develop through the embryonic stages and the larvae hatch at depth (Quetin and Ross 1984). They then start what is called the "developmental ascent" (Marr 1962), swimming up to the surface. On ascent the larvae go through three non-feeding stages, nauplius I, II and metanauplius (Fraser 1936; Mauchline and Fisher 1969), by moulting. Within 30 days after hatching, the larvae reach the top 200m of the water column as calyptopis I, the first feeding stage (Nicol 2006). By this time they have used up most of their egg reserves, and food of adequate quality and quantity is needed within six days to survive their first winter (Ikeda 1984; Hagen *et al.* 2001; Atkinson *et al.* 2002; Quetin *et al.* 2003; Nicol 2006; Meyer *et al.* 2009). Despite the omnivorous nature of Antarctic krill, it is well documented that sea-ice algae constitute an essential part of their diet, especially for juveniles in their first winter. This dependency is reflected by the strong correlation of krill recruitment success to timing and extent of winter sea-ice (Kawaguchi and Satake 1994; Loeb and Siegel 1995; Loeb *et al.* 1997; Atkinson *et al.* 2002; Frazer *et al.* 2002; Quetin *et al.* 2003; Ju and Harvey 2004). Their inability to store energy or starve makes larval krill

particularly vulnerable to any deterioration of food quality or availability as a possible consequence of ocean acidification (Kurihara *et al.* 2004b; O'Brien *et al.* 2011). This is an issue of concern since survival rates during this period of the life cycle primarily dictate the magnitude of annual recruits and eventually krill population size.

While phytoplankton nutritional quality could change with ocean acidification as discussed previously, a change in phytoplankton species composition could be equally detrimental. Krill have been found to graze predominantly on pennate diatoms and dinoflagellates (Daly 1990; Meyer *et al.* 2009). Maximum growth rates of late furcilia larvae and juveniles were recorded at the end of diatom blooms, while lowest growth rates occurred during blooms dominated by cryptophytes and prymnesiophytes (Ross *et al.* 2000). This might be in part due to the difference in size between these phytoplankton taxa. A recurrent shift in phytoplankton community structure, from diatoms (15 - 270µm) to cryptophytes (<10µm), along the Antarctic Peninsula, associated with regional warming and an increasing abundance of salps, was reported by Moline *et al.* (2004). Such changes in the phytoplankton community to a size range less suitable for krill, which cannot efficiently graze cells smaller than 6µm (Smetacek *et al.* 2004), could be the underlying reason for a rise in salps populations, which can graze particles smaller than 1µm (Le Fevre *et al.* 1998). A regime shift from krill to salp dominance could have major flow – on effects for the rest of the ecosystem (Moline *et al.* 2004).

Krill populations are already declining significantly in some areas due to changes to sea ice dynamics (Atkinson *et al.* 2004; Quetin *et al.* 2007; McClintock *et al.* 2008) and regional changes in phytoplankton densities (Montes-Hugo *et al.* 2009). Yet krill populations are facing another threat: krill fishery will likely grow due the development of new and easier harvesting technologies and enhanced demand for krill oil and other products emerging (Nicol *et al.* 2012). Therefore it is essential to understand and accurately predict any climate change – induced impacts on krill population size to set sustainable catch rates before enhanced harvest of this species begins.

Research on krill under high ocean pCO_2 scenarios is, however, in its infancy. Direct effects of elevated pCO_2 and decreased pH on krill egg and larval development, as well as adult feeding and nutrient excretion rates are being examined (Kawaguchi *et al.* 2011; Saba *et al.* 2012; Kawaguchi *et al.* 2013). To date there have been no experimental studies on the indirect effects of ocean acidification on larval krill through changes in their food, i.e. phytoplankton. To be able to predict changes in the food quality and / or quantity of Antarctic phytoplankton under different ocean acidification scenarios we need to understand whether any change will be predominantly within the biochemistry of individual species and / or through shifts in species abundance and community composition.

1.6. Aims and hypothesis

As outlined in detail in the introduction, there are several ways in which phytoplankton species can be affected by ocean acidification. Increased availability of CO_2 and lowered seawater pH can alter growth rates (Kim *et al.* 2006; Wu *et al.* 2010), change photophysiology (Wu *et al.* 2010; McCarthy *et al.* 2012), affect calcification (Riebesell *et al.* 2000; Feng *et al.* 2008; Iglesias-Rodriguez *et al.* 2008; Barcelos e Ramos *et al.* 2010; Beaufort *et al.* 2011) and change biochemical composition (Fu *et al.* 2007; Rossoll *et al.* 2012). For some species the changes are positive, for others they constitute a competitive disadvantage, potentially leading to shifts in the species composition (Kim *et al.* 2006; Paulino *et al.* 2008). Whether ocean acidification will affect the abundance of phytoplankton species and / or the biochemistry of individual species, possible changes to their physiology and biochemistry have the potential to affect grazers (Rossoll *et al.* 2012; Schoo *et al.* 2013) and predators through trophic interaction. Based on this, the question I posed for my research project was whether ocean acidification can affect the key species of the Antarctic food web, *Euphausia superba*, through impacts on phytoplankton. For this a number of fundamental questions have to be answered:

a) Are Antarctic phytoplankton species susceptible to ocean acidification?
b) If so, in what way will ocean acidification affect Antarctic phytoplankton biochemistry?
c) Can these changes in individual phytoplankton species negatively affect grazing Antarctic krill?

d) Will Antarctic phytoplankton community structure change due to ocean acidification?
e) How will potential changes in the biochemistry of individual phytoplankton species together with phytoplankton community shifts affect the nutritional quality of the whole phytoplankton community as food for krill?
f) Do Antarctic krill have the ability to detect the changes in their food and adapt if necessary?

To lay the foundations for this research topic, I focused on the specific questions outlined under a) – c). The hypothesis for this research project was that elevated pCO_2 can alter the biochemical composition of Antarctic phytoplankton species in a way that may render them inferior as food for Antarctic krill larvae during their first feeding stages. While there have been studies into the effects of elevated pCO_2 on aspects of the nutritional quality of phytoplankton species for grazers, the novelty of this work lies in the suite of biochemical parameters measured simultaneously and the choice of ecologically relevant phytoplankton species.

To address the research questions, a suitable phytoplankton incubation system to accommodate Antarctic temperature and light conditions and maintain stable seawater carbonate chemistry, had to be designed and manufactured. The effects of elevated pCO_2 on single species phytoplankton cultures and the flow – on effects on Antarctic krill larvae were first investigated by means of semi – continuous cultures (Chapters 2 and 3). After limitations of this

experimental approach were identified, a continuous culture system was developed (Chapter 4) and used to investigate the susceptibility of three Antarctic phytoplankton species to elevated CO_2 concentrations (Chapter 5). Finally, the question on how quickly phytoplankton biochemistry changes once the algae are exposed to a different pCO_2 environment was addressed in a short 48h experiment (Chapter 6). Chapter 7 summarizes the findings of this research project, puts them in perspective with the current literature and provides recommendations for future research.

The appendix provides details on the development of the semi – continuous cultures system, its limitations and a comparison between semi – and continuous culture system. Limitations of the semi - continuous culture experiments are summarized in the appendix, along with a description of a flow – through system, used for the final experiment with the diatom *Pseudo-nitzschia subcurvata* and Antarctic krill juveniles. Although the experiment was unsuccessful, suggestions for future research and possible explanations for the failure are provided.

2. Pilot study into the effects of elevated pCO_2 on the diatom *Synedropsis hyperborea* and the flow – on impacts on grazing Antarctic krill larvae

2.1. Abstract

The hypothesis for this research project was that elevated pCO_2 can alter the biochemical composition of Antarctic phytoplankton species in a way that may render them inferior as food for Antarctic krill larvae during their first feeding stages. Therefore, the aim of this experiment was to investigate the effects of ocean acidification on phytoplankton as food for krill.

For this a culture system had to be designed that could maintain phytoplankton growing exponentially for extended periods of time at different pCO_2. The phytoplankton grown at the different pCO_2 could then be fed to krill larvae over a time period sufficient to detect physiological changes in the larvae. Based on best practice guides to ocean acidification experiments and my own pilot studies, I decided to manipulate the phytoplankton medium via the addition of CO_2 gas in a semi – continuous culture system.

A pilot study to assess the effects of elevated pCO_2 on the diatom *Synedropsis hyperborea* and flow – on impacts on grazing Antarctic krill larvae did not reveal strong CO_2 perturbations in the alga or the larvae feeding on it. While cellular carbohydrate concentrations increased with elevated CO_2 concentrations, pCO_2 did not significantly alter C:N ratio or pigment composition in *Synedropsis hyperborea* and fatty acid profile did not show a conclusive pattern. In line with the weak CO_2-signal in the alga, daily mortality rates of the krill larvae did not follow a clear trend. Larval mortality rate was highest amongst larvae feeding on algae grown at 264 and 527µatm.

Total lipid content per dry weight was highest in larvae feeding on alga grown at 726µatm, while those larvae also had the highest percentage of carbon per dry weight and polyunsaturated fatty acids and lowest ratio of the essential polyunsaturated fatty acids 22:6ω3 : 20:5ω3. In conclusion the biochemistry of the Southern Ocean diatom *Synedropsis hyperborea* appears to be little affected by an increase in pCO_2 up to 726µatm CO_2 and as a consequence there were no clear flow – on effects on the development of larval Antarctic krill feeding on the alga. This pilot study served as a valuable learning experience and limitations and ways to address these in following experiments are discussed.

2.2. Can ocean acidification affect Antarctic krill through a pCO_2 – induced change in phytoplankton biochemistry?

The hypothesis for this research project was that elevated pCO_2 can alter the biochemical composition of Antarctic phytoplankton species in a way that may render them inferior as food for Antarctic krill larvae during their first feeding stages. This was based on literature reports of CO_2 – induced changes in phytoplankton C:N:P, cellular carbohydrate and polyunsaturated fatty acid concentrations (Bellerby *et al.* 2008; Iglesias-Rodriguez *et al.* 2008; Paulino *et al.* 2008; Thornton 2009; Rossoll *et al.* 2012; Schoo *et al.* 2013). It has also been shown that the biochemistry of phytoplankton and changes therein can affect grazer growth and reproduction (Rothhaupt 1995; Urabe *et al.* 2003; Chen *et al.* 2012; Rossoll *et al.* 2012; Schoo *et al.* 2013). In this context, phytoplankton fatty acid profile is of particular interest as an indicator of their nutritional quality for grazers. Fatty acids are divided into polyunsaturated fatty acids (PUFA), monounsaturated fatty acids (MUFA) and saturated fatty acids (SFA). The composition of fatty acids, most importantly PUFA, are critically important for grazer development, reproduction and hatch rates, as has been well researched by the aquaculture industry amongst others (Koven *et al.* 1989; Harrison *et al.* 1990; Carvalho and Malcata 2005; Bell *et al.* 2007; Yoshida *et al.* 2011; Chen *et al.* 2012).

2.3. Manipulation of the carbonate chemistry – method appraisal

To better understand and predict the impact of ocean acidification on organisms, experimental research must simulate natural changes in ocean chemistry. Due to the recent emergence of this field of science, different methods for manipulation of seawater chemistry have been applied. Comparison of the results between studies has therefore been difficult at times (cf. Riebesell *et al.* 2000; Iglesias-Rodriguez *et al.* 2008). There are major differences in how the various methods alter the carbonate chemistry and in the following I will discuss these and outline the currently recommended methods of choice for ocean acidification experiments.

2.3.1. Acid – base addition: lowered pH and alkalinity, equal dissolved inorganic carbon

Adding acid (HCl) to seawater lowers the pH and total alkalinity, however, it does not increase dissolved inorganic carbon (DIC), i.e. the sum of CO_2, H_2CO_3, CO_3^{2-} and HCO_3^- concentrations. The simplicity, cost effectiveness and / or lack of knowledge about pH effects on other components of the seawater carbonate system at the time made this (adding acid / base (NaOH) to seawater) the method of choice to achieve the desired pH in early ocean acidification experiments (Burkhardt and Riebesell 1997; Burkhardt *et al.* 1999; Riebesell *et al.* 2000; Langer *et al.* 2006). Acid / base adjustment is still a valid experimental approach if the effects of lowered pH are being investigated independently of any other parameters of the carbonate system.

However, for most experiments involving autotrophic or calcifying organisms, this method is now deemed to be inadequate in simulating natural conditions under future ocean acidification (Riebesell *et al.* 2010).

2.3.2. CO_2 addition: lowered pH, equal alkalinity, increased dissolved inorganic carbon

With a greater understanding of the interdependency of pH, total alkalinity and DIC, a new form of ocean acidification experiments emerged from around the early 2000s (cf. Tortell *et al.* 2002; Collins and Bell 2004; Kim *et al.* 2006). Pre-mixed CO_2-air gases of defined CO_2 concentration were bubbled into the seawater of experimental units (Riebesell *et al.* 2007; Iglesias-Rodriguez *et al.* 2008). With this approach, pH, total alkalinity and components of the carbonate system respond in the same way as they would do in natural systems. At a pH of 7.5, the acid / base method results in a 23% and 22% lower pCO_2 and HCO_3^- concentration, respectively (Fig. 2.1) (Hurd and Hepburn 2009).

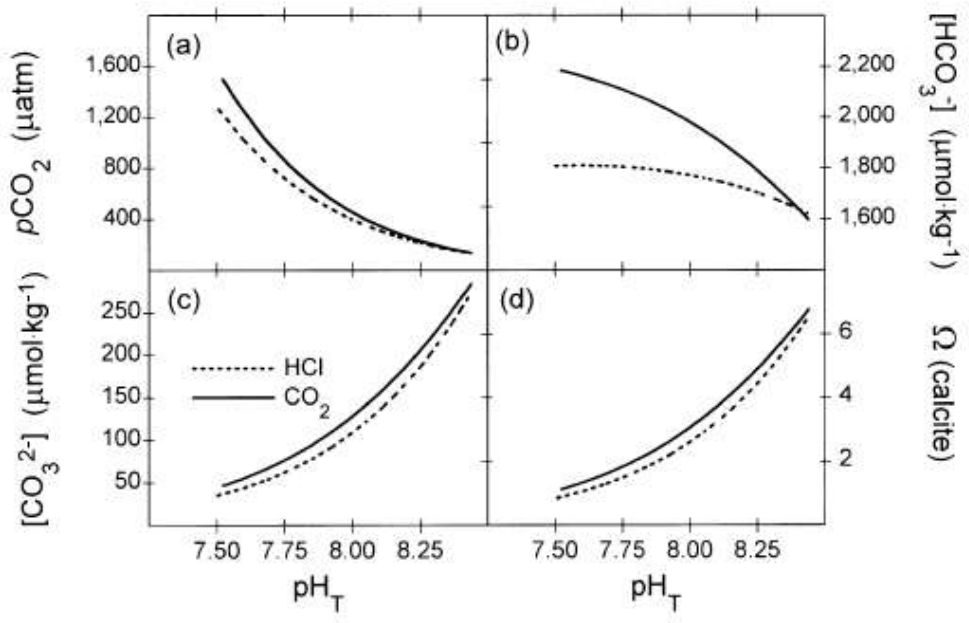

Figure 2.1 Theoretical CO_2 parameters as a function of seawater pH. The four graphs show the difference of acidifying (dotted line) and CO_2 addition (bold line) on CO_2 parameters in seawater: a) equilibrium CO_2 partial pressure, b) bicarbonate ion concentration, c) carbonate ion concentration and d) calcite saturation index Ω (from Hurd and Hepburn 2009).

Some experiments with phytoplankton cultures have shown that fragile species are negatively affected by direct bubbling of the medium through small-scale turbulence, resulting in reduced growth rates and / or biomass (Hurd and Hepburn 2009; Shi *et al.* 2009). Flocculation of dissolved organic matter, caused by the perturbation resulting from CO_2 aeration, can also stimulate bacterial growth (Riebesell *et al.* 2008). Therefore, the cheap, simple and non-invasive adjustment of pH by addition of acid was improved to facilitate natural changes in carbonate chemistry by adding equimolar concentrations of HCl and $NaHCO_3$. Addition of $NaHCO_3$ solution neutralizes the decrease in total alkalinity and supplies additional DIC to match the effect of CO_2 introduction (Hurd and Hepburn 2009; Schulz *et al.* 2009). One

drawback of this method is the constant need to monitor the carbonate system to allow for adjustments.

Another way to avoid the negative effects of bubbling is to adjust the culture medium first to the chosen carbonate chemistry conditions and then inoculate with the culture. Alternatively, Poers *et al.* (2010) used low density polyethylene bags filled with a concentrated carbonate buffer of elevated pCO_2 and inserted them as a CO_2 reservoir directly into culture vessels. The characteristics of the bag membrane allow for a constant release of CO_2 into the surrounding culture medium. Both methods, however, make further adjustments or maintenance of pCO_2 levels during the experiment impossible. Under such circumstances pCO_2 levels have to be monitored closely and cell densities need to be kept low to avoid substantial draw-down of CO_2 through photosynthesis and thereby significant deviations from the desired pCO_2 levels. Moreover, any build-up of waste products such as O_2 cannot be avoided in closed systems like these.

Akin to bubbling CO_2-gas directly into the seawater, CO_2 – gas can instead be flushed into the headspace of the experimental culture vessel. The CO_2 concentration in the headspace and culture medium equilibrate over time, which results in the same realistic manipulation of the seawater carbonate system and pH, but without the harmful small-scale turbulence occurring through gas bubbles. Gentle agitation of the culture vessels increases the diffusion rate of CO_2 from the headspace into the medium. With a constant supply of defined CO_2-air mix into the headspace, deviations from the desired

pCO_2 through photosynthesis due to high cell densities can be delayed. Build-up of O_2 from photosynthesis is prevented as the headspace is constantly renewed, which makes this approach advantageous over closed systems. To avoid the expense of using pre-mixed CO_2 – air gases, inexpensive CO_2 gas can be mixed with air to the desired pCO_2 concentrations.

2.4. The effects of elevated pCO_2 on the diatom *Synedropsis hyperborea* and flow – on impacts on grazing Antarctic krill larvae

Preliminary trials to establish the most effective way to manipulate the pCO_2 of large phytoplankton culture volumes and methodological considerations for Antarctic krill feeding experiments with these high CO_2 – grown cultures are discussed in Chapter I of the Appendix.

Two separate feeding experiments were conducted with the diatoms *Synedropsis hyperborea* and *Pseudo-nitzschia subcurvata* and Antarctic krill larvae. The first experiment with *Synedropsis hyperborea* cultures served as a pilot study for the second experiment, and based on experience gained from this experiment minor adjustments were made for the *Pseudo-nitzschia subcurvata* study (Chapter 3).

Synedropsis hyperborea is a small diatom that was dominant at elevated pCO_2 treatments in minicosm experiments conducted in Antarctica (Andrew Davidson pers. comm.). Here I report the effects of elevated pCO_2 concentrations on the biochemistry of this Antarctic diatom incubated under

simulated macronutrient and light conditions similar to which it was isolated from (Davis Station, Antarctica) and the impacts on Antarctic krill larvae.

2.4.1. Methods

2.4.1.1. Incubating phytoplankton cultures at various pCO_2 levels

Details on the semi-continuous culture setup used for this experiment can be found in Chapter 3 and Chapter I of the appendix. Phytoplankton cultures were grown in f/2 medium (Guillard and Ryther 1962; Guillard 1975), where nitrate, phosphate and silicate levels were adjusted to concentrations reported around O'Gorman Rocks, off Davis Station, Antarctica (Gibson 1998; Roden *et al.* 2013). I chose f/2 medium as it lacks any buffer, that could affect the carbonate chemistry and trace metal speciation in the growth medium and would thereby affect medium pH and phytoplankton growth (Shi *et al.* 2009). Experimental conditions are listed in Table 2.1.

pCO_2 concentrations were calculated with the CO2SYS.BAS Excel programme (Lewis and Wallace 1998), using the constants after Mehrbach *et al.* (1973) as refitted after Dickson and Millero (1987) and based on pH, total alkalinity, salinity, temperature and nutrient concentrations.

The phytoplankton cultures were acclimated to the elevated pCO_2 media for at least nine generations and then sampled for analysis of carbon to nitrogen ration (C:N), carbohydrate, and fatty acid and lipid class composition. Cell

density was measured daily to express biochemical parameters on a per cell basis where appropriate.

Table 2.1 Experimental conditions for phytoplankton cultures and krill larvae. Values are averages with standard deviations (SD) in brackets and number of samples (n), if applicable.

Species	Acclimation [number of generations and days]	Light intensity [µmol m^{-2} s^{-1}] (±SD)	Average nutrient concentrations [µM] of fresh medium (±SD) (n=4)		
			NO_x (NO_3^- & NO_2^-)	Si	P
Synedropsis hyperborea	9 – 15 generations, 10 days	154 (±3)	24 (±2)	81 (±0.5)	6 (±4)

	CO_2 concentration [µatm] (±SD)			
Synedropsis hyperborea	158 (±61)	264 (±91)	527 (±93)	726 (±81)
Krill	301 (±50)	345 (±66)	367 (±72)	375 (±82)

2.4.1.2. Feeding krill larvae phytoplankton grown at elevated pCO_2

Adult krill were caught in early 2010, in East Antarctica (66°S, 73°E), (*Synedropsis hyperborea* experiment) and maintained for experiments and breeding in the laboratory. Krill larvae were reared from eggs spawned from these adults in the laboratory. Once the larvae reached calyptopis I, they were fed an algal mixture of *Geminigera cryophila*, *Pyramimonas gelidicola* and *Phaeodactylum tricornutum*, along with freshly hatched *Artemia* nauplii. The experiment was started with animals entering the calyptopis II stage and lasted 44 days to cover at least three moult events. To minimize confounding effects caused by the variation in quality of larval egg batches, I pooled larvae that

hatched within days of each other from several females. Larvae that hatched from several egg batches were picked randomly and distributed into 5L clear plastic containers filled with the phytoplankton culture of each CO_2 treatment. Each of the CO_2 treatments was replicated by three 5L containers with between 33 and 37 animals in each container. The 5L containers were floated in krill holding tanks, chilled to 0.5°C. Details of the experimental setup are described in Chapter 3 and Chapter I of the Appendix.

Prior to transferring krill larvae into the phytoplankton cultures, the pH of cultures subjected to elevated pCO_2 were raised to ambient ocean pH to avoid confounding pH effects on krill larvae by changing the water chemistry. The carbonate chemistry of the krill container was determined with the CO2SYS.BAS Excel programme with these pH measurements, alkalinity samples taken from the phytoplankton culture and the seawater used for mixing.

Moults and dead animals were collected as part of the daily inspection and preserved in 5% formalin-seawater.

2.4.1.3. Physiological and chemical analyses

For details on analyses of phytoplankton growth rates, cell dimensions, C:N ratio, cellular carbohydrate concentrations and lipid, fatty acid and pigment composition I refer the reader to the methods section of Chapter 3. Equally, a

detailed description on the methods for measuring larval growth and mortality can be found in Chapter 3.

2.4.1.4. Statistical analysis

Phytoplankton

Due to the large scale of the experiment it was not practical to replicate culture bags for each CO_2 concentration. To get an estimate of sampling and analysis error, I chose to subsample each culture in triplicate per sampling date. However, these do not represent true replication, nor do repeated sampling dates. Bag to bag variations are therefore confounded with treatment effects. Statistical analysis and any conclusions drawn from these experiments have to be considered accordingly. Individual biochemical results of the phytoplankton samples were analysed with generalized additive models (GAM) (Wood 2006), smoothing over CO_2 concentrations, with separate splines per day. This allows for changes between the sampling days, while keeping the focus on the trend in results with increasing CO_2 concentration. Cell volume and length differences were determined by analysis of variance (ANOVA).

Krill

Krill results were analysed with linear regression models, except for the mortality data. A proportional hazards Cox model was fitted to the daily mortality rates of krill fed *Synedropsis hyperborea*.

2.4.2. Results

2.4.2.1. *Synedropsis hyperborea*

Phytoplankton cell size and growth rate

Synedropsis hyperborea showed no significant difference in growth rate post acclimation, however, the volume of cells grown at 527µatm was significantly larger than at other CO_2 concentrations ($p<0.001$, data not shown).

Total lipid, lipid class and fatty acid concentrations

The GAM model did not detect a statistically significant trend in total lipid concentration per cell nor for lipid classes for *Synedropsis hyperborea*.

Increasing CO_2 concentrations had no effect on polyunsaturated fatty acid (PUFA) concentrations in *Synedropsis hyperborea* (Fig. 2.2).

Docosahexaenoic acid (DHA, 22:6ω3) and Eicosapentaeonic acid (EPA, 20:5ω3) are the two main long-chain ($>C_{20}$) PUFA important for krill nutrition (Hagen *et al.* 2001). DHA and EPA concentrations per cell were not significantly affected by pCO_2 in *Synedropsis hyperborea*.

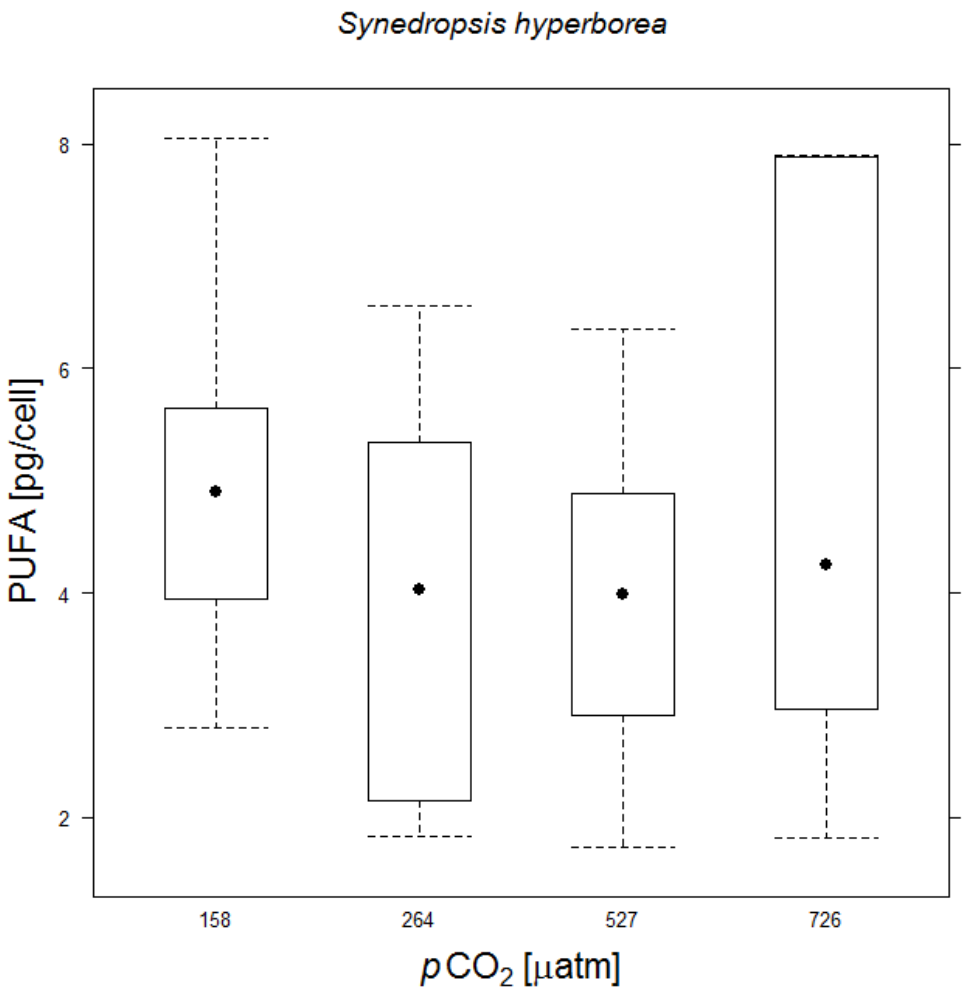

Figure 2.2 Polyunsaturated fatty acid (PUFA) content per cell [pg/cell] for *Synedropsis hyperborea*. Data points are medians, boxes cover the lower and upper quartiles and the whiskers delineate the lowest and greatest values.

Cellular carbohydrates concentration

Cellular particulate carbohydrate (CHO) concentration was positively affected by increased CO_2 concentration in *Synedropsis hyperborea* (Fig. 2.3). The GAM model showed a statistically significant trend on days 13, 33 and 41 for *Synedropsis hyperborea* (* $p<0.05$).

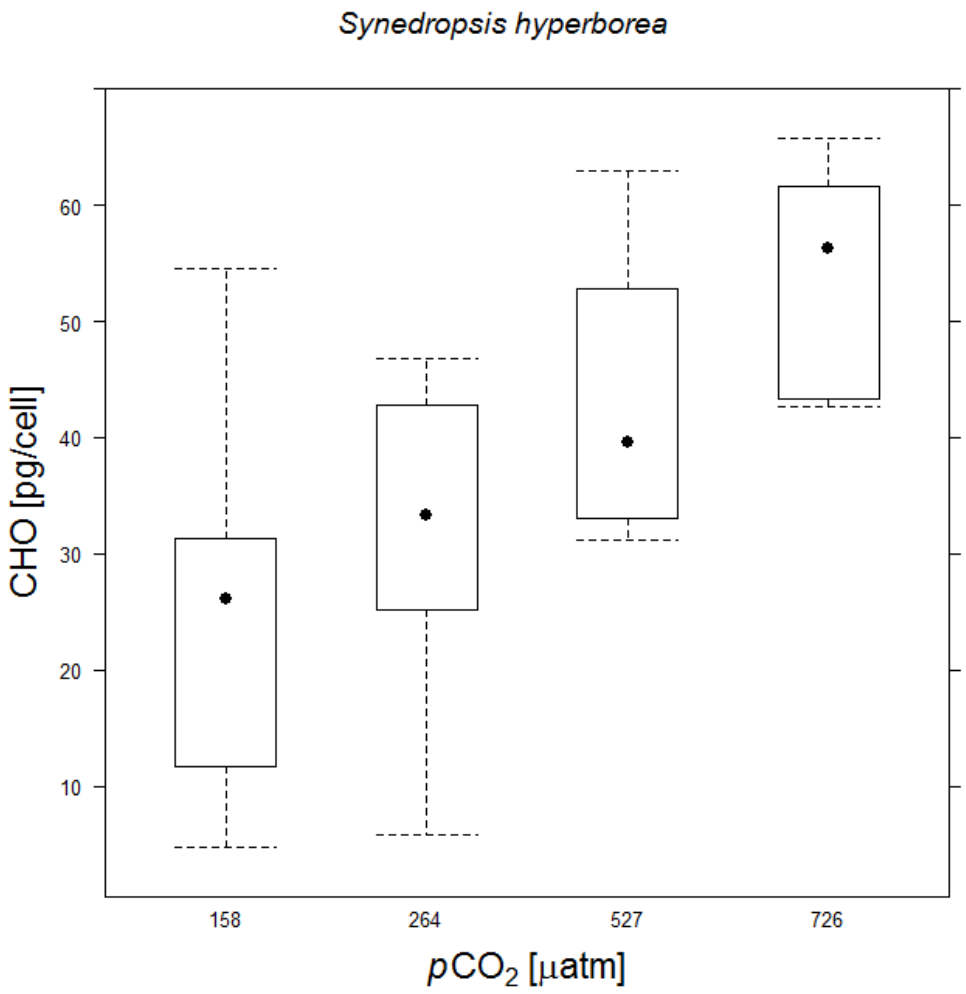

Figure 2.3 Increasing carbohydrate content per cell [pg/cell] of *Synedropsis hyperborea* cultures with increasing pCO_2. Data points are medians, boxes cover the lower and upper quartiles and the whiskers delineate the lowest and greatest values.

Carbon to nitrogen ratio

There was no clear trend in cell-specific carbon (C), nitrogen (N) (data not shown), percent carbon of dry mass (%C), percent nitrogen of dry mass (%N) or C:N in *Synedropsis hyperborea* (Table 2.2).

Table 2.2 Elemental composition of *Synedropsis hyperborea* in % dry mass. Results are averages over the experiment. Standard deviation in brackets and n = number of samples, with each krill sample containing between 2 and 3 larvae.

Synedropsis hyperborea	**%C**	**%N**	**C:N**
158µatm n = 15	5.1 (4.0)	0.5 (0.1)	9.8 (5.5)
264µatm n = 14	3.9 (1.2)	0.4 (0.1)	8.7 (1.1)
527µatm n = 18	5.1 (2.2)	0.5 (0.2)	10.4 (3.7)
726µatm n = 18	4.4 (1.3)	0.5 (0.1)	9.6 (2.1)

2.4.2.2. Krill larvae

Larval development

There was no statistically significant difference between dry weight or telson length of larvae fed *Synedropsis hyperborea* cultures, irrespective of the pCO_2 at which the alga was grown. This means that there was no difference in larval growth between treatments. Intermoult periods, the number of days between moulting, were similar for all treatments and ranged between 10 and 12 days (data not shown), which means there was no developmental delay as a result of the different treatments.

Total lipid, lipid class percentages and fatty acid concentrations

Krill fed *Synedropsis hyperborea* culture grown at 726µatm CO_2 had significantly higher total lipid content per dry weight larvae, higher percentages of total lipid of triacylglycerol (TAG) and lower percentages of total lipid of polar lipid (PL) ($p<0.05$) (Table 2.3).

Table 2.3 Lipid content (µg/mg) and lipid class composition (as percent of total lipid) in krill larvae. Standard deviation in brackets, n= sample number, each sample contained between 6 and 12 larvae. *denotes statistical significance $p<0.05$

	Krill larvae		
n=3	**%TAG**	**%PL**	**Total lipid content per dry weight [µg/mg]**
158µatm	13.0 (4.9)	80.9 (6.6)	69.3 (9.7)
264µatm	14.4 (3.2)	77.9 (1.7)	63.7 (13.6)
527µatm	11.6 (3.3)	82.4 (6.2)	60.5 (6.7)
726µatm	23.7 (1.9) *	71.6 (2.3) *	92.7 (3.9) *

The DHA:EPA ratio in krill larvae feeding on phytoplankton cells grown at 726µatm significantly decreased, while concentrations of monounsaturated fatty acids (MUFA), ω6 PUFA, ω3 PUFA and total PUFA increased with increasing pCO_2 of the phytoplankton growth medium (Table 2.4).

Table 2.4 Fatty acid concentrations in µg per mg dry weight and DHA:EPA ratio for krill larvae feeding on *Synedropsis hyperborea* cells grown at CO_2 concentrations of 158µatm, 264µatm, 527µatm and 726µatm. Standard error in brackets and n = number of samples, with statistical significance * $p < 0.05$, ** $p < 0.01$, *** $p < 0.005$

Fatty acid [µg/mg]	**MUFA**	**ω6 PUFA**	**ω3 PUFA**	**Total PUFA**	**DHA : EPA**
158µatm n = 3	14.9 (0.7)	4.6 (0.3)	20.5 (2.4)	25.7 (2.4)	0.13 (0.00)
264µatm n = 3	21.4 (1.8)*	5.8 (0.9)	21.8 (2.4)	28.3 (3.4)	0.13 (0.01)
527µatm n = 3	15.8 (1.8)	5.0 (0.8)	19.4 (2.8)	25.0 (3.8)	0.13 (0.01)
726µatm n = 3	24.8 (4.0)***	6.9 (0.9)*	25.5 (2.7)*	33.1 (3.5)*	0.10 (0.01)***

Carbon to nitrogen ratio (C:N)

Although there was no clear trend in the elemental composition of the diatom *Synedropsis hyperborea*, krill larvae fed the highest pCO_2 grown diet had slightly increased %C and C:N (Table 2.5).

Table 2.5 Elemental composition of krill larvae in % dry mass. Results are from whole animals at the end of the experiment. Standard deviation in brackets and n = number of samples, with each krill sample containing between 2 and 3 larvae. * statistical significance $p < 0.05$

Krill fed *Synedropsis hyperborea*	**%C**	**%N**	**%S**	**C:N**
158µatm n = 7	36.0 (1.0)	10.2 (0.7)	1.1 (0.1)	3.5 (0.2)
264µatm n = 6	36.4 (1.6)	10.2 (0.4)	1.1 (0.1)	3.6 (0.3)
527µatm n = 7	36.2 (1.2)	9.9 (0.5)	1.1 (0.1)	3.7 (0.1)
726µatm n = 8	37.5 (0.8) *	10.2 (0.3)	1.1 (0.1)	3.7 (0.2) *

Mortality

There was a significantly higher daily mortality rate among krill larvae fed *Synedropsis hyperborea* grown at 264 and 527µatm than those krill fed *Synedropsis hyperborea* grown at 159 and 726µatm (Fig. 2.4) ($p<0.05$). Figure 2.8 depicts daily mortality rates as calculated as ratio of sum of dead larvae to sum of live larvae per treatment. The thick black line represents the modelled daily mortality rates together with twice the standard error of the difference as upper and lower confidence bounds (thin black lines) to determine statistical significant at an approximate 95% level of probability.

In all treatments the highest daily mortality rates occurred during the time of larvae moulting from calyptopis II into the next larval stage, calyptopis III.

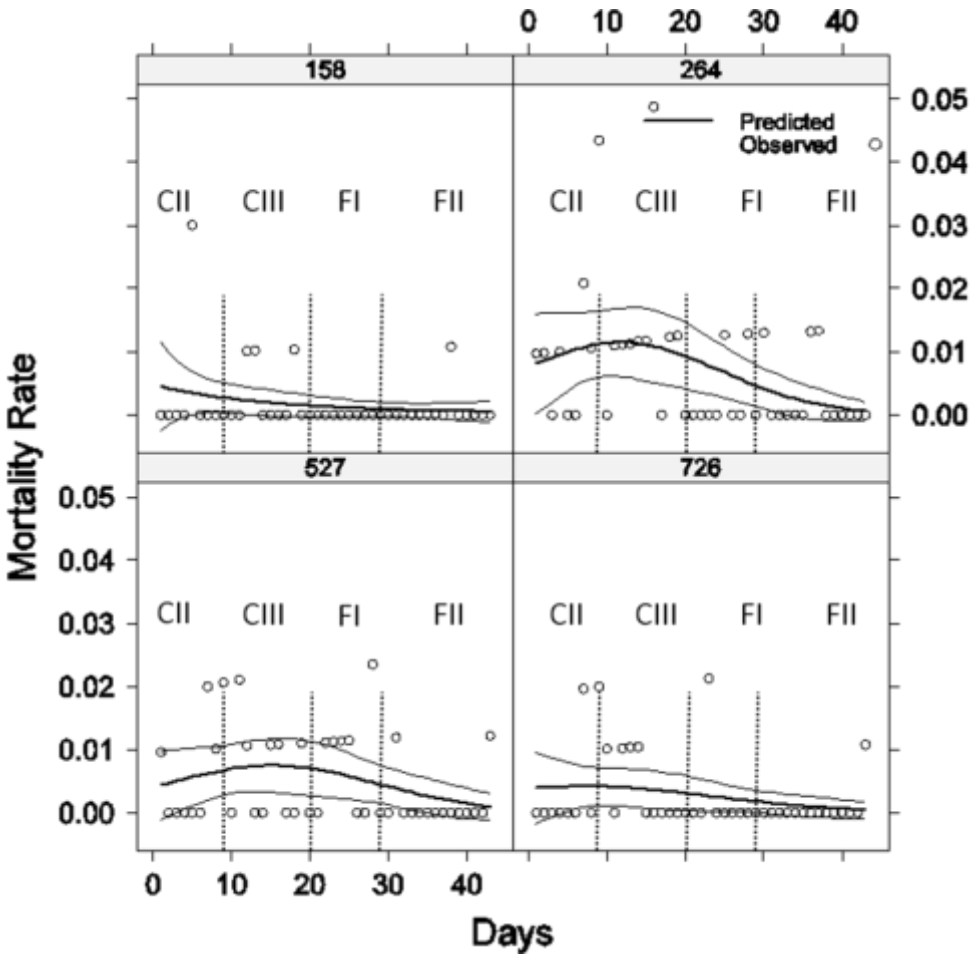

Figure 2.4 Daily mortality rate for krill larvae fed *Synedropsis hyperborea* cells grown at 158µatm, 264µatm, 527µatm and 726µatm CO_2. Dashed vertical lines indicate the approximate timing of metamorphoses between larval stages. Circles represent the ratio of sum of dead larvae to sum of live larvae per treatment; thick black line represents the modelled daily mortality rates together with twice the standard error of the difference as upper and lower confidence bounds (thin black lines) to determine statistical significant at an approximate 95% level of probability.

2.4.3. Discussion

Overall the diatom *Synedropsis hyperborea* appeared tolerant to changes in pCO_2 under experimental conditions used in this study. Increased CO_2 concentrations did not result in significant changes in C:N ratio and pigment composition or concentration of *Synedropsis hyperborea* and fatty acid profile followed no conclusive trend.

While I measured cellular carbohydrate concentrations in *Synedropsis hyperborea*, which increased, as seen elsewhere under low pH conditions (Thornton 2009), I did not monitor or quantify excretion of exopolymer saccharides. It is possible that C:N uptake rates were influenced by CO_2 concentrations, but that the C:N ratio was maintained through excretion of excess C as exopolymer saccharides. Engel (2002) reported increased production of exopolymer particles at elevated CO_2 concentrations and CO_2 uptake in natural phytoplankton communities of the Baltic Sea. Burkhardt *et al.* (1999) showed that phytoplankton C:N ratio is influenced by CO_2 concentration, however, similar to our findings, beyond ambient pCO_2 there was little change in C:N with increasing CO_2 concentration in their experiments.

As expected from the lack of a clear CO_2-signal in the alga, there was no clear trend in the daily mortality rates of the krill larvae. The highest daily mortality rates occurred in the transition phase into and during larval stage calyptopis III amongst all treatments. This spans across days 10 to 20 of the experiment. It is unclear from our results whether the timing of the peak mortality is

associated with the respective larval stage or whether it is a consequence of experimental stress on the animals. Peak daily mortality occurred during a similar time frame (days 10 – 20) during the second experiment with krill larvae and the diatom *Pseudo-nitzschia subcurvata* (Wynn-Edwards *et al.* submitted, Chapter 3). Yet contrary to the experiment described here, during the second experiment krill larvae were within larval stage calyptopis II and moulting into larval stage calyptopis III. This would suggest that peak mortality is not associated with a specific larval stage but a natural consequence of the experiment stress when larvae are transferred from their rearing environment to that of the experiment with all the handling that this involves.

Lowest mortality rates were found in larvae feeding on alga grown at 158 and 726µatm. Those larvae also had the highest lipid content per dry weight, yet this was not reflected by the lipid content of the algal cultures. I did not measure krill filtration rate in our experiment and therefore I cannot rule out that krill larvae feeding rate on 158 and 726µatm CO_2 – grown algae was higher than that of the other two treatments and that the larvae were therefore able to accumulate more lipids which in turn reduced their mortality rate compared to larvae feeding on algae grown at 264 and 527µatm CO_2. Abiotic factors such as phosphorus limitation have been shown to increase cell wall thickness of the green alga *Chlamydomonas reinhardtii* with a subsequent reduction in grazing rate (Van Donk *et al.* 1997). Yet, increased temperature and lowered pH affected the palatability of the seaweed *Sargassum linearifolium* to the effect of increased feeding rates by the amphipod

Peramphithoe parmerong (Poore *et al.* 2013) and CO_2 concentration significantly affected the occurrence of cell chain formation of the Southern Ocean diatom *Proboscia alata* (Hoogstraten and Timmermans 2012). Changes in the morphology of *Synedropsis hyperborea* cells in the intermediate CO_2 treatment was not measured in this experiment but could have affected grazing rates by krill larvae and thus could account for the difference in lipid accumulation between krill larvae. Grazing rates are difficult to establish in krill larvae (pers. comment So Kawaguchi) but based on these findings should be a consideration for future experiments.

2.4.4. Conclusion

Although this experiment did not reveal strong CO_2 perturbations in the alga or the larvae feeding on it, it served as a valuable learning experience. The main concern with this experiment was that the actual CO_2 concentrations in the phytoplankton cultures were much lower than intended. The lowest CO_2 treatment was meant to be at approximately atmospheric levels (390ppm), but was found to average around 158µatm. The highest CO_2 treatment was bubbled with CO_2-enriched air of approximately 950ppm, but the culture medium of that treatment averaged around 726µatm. Although cell densities were low due to the low nutrient concentrations, around 40,000cells/ml, photosynthetic activity outweighed the re-supply of CO_2 obtained by bubbling the cultures. CO_2 adjustment via bubbling is a logistically simple and relatively cheap way of maintaining stable carbonate chemistry. However, due to the potential damage to fragile cells, bubbling speed, i.e. the volume of

CO_2 – enriched air delivered per minute, cannot be increased substantially to accommodate large culture vessels. Thus, instead of increasing the volume of air bubbling through the cultures, the volume of CO_2 added to the air can be increased. Since the culture pH was monitored daily, regular adjustments of the volume of CO_2 added to the air bubbling through the cultures was used to address this issue in the following experiment (Chapter 3).

3. Can increased pCO_2 alter the biochemistry of Antarctic phytoplankton and thus affect the survival of Antarctic krill larvae?

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3.1. Abstract

Anthropogenic CO_2 emissions are continuously absorbed by the oceans, thereby raising seawater pCO_2, potentially altering the biochemistry and physiology of the phytoplankton on which grazers depend. Little is known about the potential for increased pCO_2 to affect the nutritional quality of Antarctic phytoplankton and the flow on effects on Antarctic krill, *Euphausia superba*, a key species in the food web. We examined phytoplankton growth rates, lipid class and fatty acid profile, carbohydrate content and elemental composition of the Antarctic diatom *Pseudo-nitzschia subcurvata* grown at a range of CO_2 concentrations (326µatm – 896µatm). Larval krill (calyptopis I to furcilia I) mortality and biochemistry were measured after feeding on the diatom for over four weeks. Changes in the phytoplankton were subtle and included a decrease in long-chain ($\geq C_{20}$) polyunsaturated fatty acids (22:6ω3, DHA and 20:5ω3, EPA), likely due to lowered pH, and a small increase in carbohydrate concentrations, likely due to increased CO_2 concentration. While changes in the biochemistry of *P. subcurvata* were not directly reflected by changes in the biochemistry of krill larvae that survived until the end of the experiment, krill larvae fed *P. subcurvata* grown at 896µatm CO_2 had significantly higher rates of mortality than those larvae fed algae grown at ambient CO_2 concentration. We conclude that CO_2-induced effects on the nutritional quality of this phytoplankton species can impair the fitness of calyptopis I to furcilia I krill larvae and diminish their survival rates.

3.2. Introduction

Elevated CO_2 concentration influences the ratio of carbon to nutrient uptake rates in phytoplankton (Burkhardt and Riebesell 1997; Riebesell *et al.* 2007; Bellerby *et al.* 2008; Paulino *et al.* 2008) and consequently C:N:P ratio (Iglesias-Rodriguez *et al.* 2008; Hoogstraten and Timmermans 2012; Schoo *et al.* 2013). Furthermore, a reduction in the percentage of polyunsaturated fatty acids (PUFA) has been reported in the diatom *Thalassiosira pseudonana* (Rossoll *et al.* 2012). CO_2 – induced alterations to the nutritional quality of phytoplankton have been shown to negatively affect grazer growth rates (Urabe *et al.* 2003; Urabe and Waki 2009; Rossoll *et al.* 2012; Schoo *et al.* 2013).

Polar species naturally experience a large range of pCO_2, so these organisms may be more resilient to extreme pCO_2 (McNeil *et al.* 2011) but little is known about their possible resilience (Fabry *et al.* 2009) and this is particularly true for Antarctic phytoplankton (Riebesell 2004; Montes-Hugo *et al.* 2009). Here we study the effects of elevated pCO_2 on the biochemical composition of the ubiquitous Antarctic diatom, *Pseudo-nitzschia subcurvata*, and whether any observed changes render them inferior as food for krill larvae during their first feeding stages. Our experiments isolate the nutritional effects of the diatom from any direct affects of lowered seawater pH on the krill larvae.

There is growing evidence that Antarctic krill are directly affected by ocean acidification. They increase feeding and nutrient excretion and show differences in catabolic pathways, consistent with increased physiological costs associated with regulating internal acid-base equilibria (Saba *et al.* 2012). Krill larval recruitment, largely driven by winter survival, is most likely the most sensitive population parameter (Flores *et al.* 2012) and rising seawater pCO_2 has been shown to threaten embryonic development and hatch rates (Kawaguchi *et al.* 2011; Kawaguchi *et al.* 2013).

Little is known about possible indirect effects of ocean acidification on Antarctic krill. However, larval development includes life history traits that make them particularly susceptible to indirect effects of ocean acidification. Females spawn in surface layers in early austral summer, generally offshore (Siegel 1988). The eggs sink to a depth of several hundred to a thousand metres (Marr 1962). During their descent the eggs develop through the embryonic stages and the larvae hatch at depth (Quetin and Ross 1984). The larvae then start what is called the "developmental ascent" (Marr 1962), during which they go through three non-feeding stages by moulting (Fraser 1936; Mauchline and Fisher 1969). The larvae reach the top 200m of the water column as calyptopis I, the first feeding stage (Nicol 2006). By this time they have used up most of their egg reserves, and food of adequate quality and quantity is needed within six days of metamorphosis into calyptopis I to survive their first winter (Ikeda 1984; Atkinson *et al.* 2002; Quetin *et al.* 2003; Nicol 2006; Meyer *et al.* 2009). Their lack of and inability to accumulate lipid stores for energy reserves may make larval krill particularly vulnerable to

deterioration of food quality or availability (Yoshida *et al.* 2009) as a consequence of ocean acidification, as shown for other organisms (Kurihara *et al.* 2004b; Kurihara *et al.* 2004a). Yoshida et al. (2011) found that hatching success was correlated with the omega – 3 long-chain ($\geq C_{20}$) polyunsaturated fatty acid 22:6ω3 (docosahexaenoic acid, DHA) as well as the ratios of DHA : EPA (eicosapentaenoic acid, 20:5ω3), and saturated fatty acids : polyunsaturated fatty acids (SFA : PUFA). They also found that DHA and EPA were quantitatively among the most utilized fatty acids between the multiple-cell stage of the embryos and the third larval stage, metanauplius. In this first study into the possible indirect effects of CO_2 – induced changes in phytoplankton as food for krill, we aimed to isolate the effects of ocean acidification on larvae from the effects caused by CO_2-affected phytoplankton food. This experimental setup does not simulate natural conditions, under which larvae and phytoplankton will be affected by increased pCO_2 simultaneously, but it is a useful precursor to full factorial experiments and will help understand to effects of each individual stressor that Antarctic krill may be subjected to under future ocean acidification.

Here we test the hypothesis that elevated pCO_2 can alter the biochemical composition of Antarctic phytoplankton species in a way that may render them inferior as food for krill larvae during their first feeding stages.

First, we studied the effects of elevated pCO_2 concentrations on the biochemistry of *P. subcurvata* under macronutrient and light conditions that simulate the environment from which it was isolated (Davis Station,

Antarctica). Current projections of the effects of enhanced pCO_2 on primary producers are commonly based on studies using nutrient replete conditions and moderate light intensities. It is now recognised that these projections are potentially flawed as nitrate assimilation rates are lower at the low temperatures that prevail in the Southern Ocean (Dugdale and Wilkerson 1991; Reay *et al.* 2001). Further, photoinhibition is a potential natural limitation under the high light intensities experienced at or near the surface in Antarctic waters.

Secondly, we examined the effect of the changed biochemistry of the alga on Antarctic krill larvae when fed the CO_2-acclimated diatom over four weeks. To our knowledge, this is the first study into the effects of CO_2-induced altered nutritional quality of phytoplankton on Antarctic krill larvae. Key biochemical parameters such as total lipid and constituent fatty acid content, in particular the key PUFA, and elemental composition were measured in the phytoplankton cultures throughout the experiment as indicators of food quality. Lipid, elemental and amino acid composition in the krill larvae were quantified at the end of the experiment as indicators of any deficiencies of the diet.

Finally, we determined survival, growth rate and intermoult period of krill larvae as indicators of their physiology.

Our results show subtle but significant responses of phytoplankton physiology to acidification that reduced the survival of krill larvae. The ecological implications of these findings are discussed.

3.3. Methods

3.3.1. Incubating phytoplankton cultures at various pCO_2 levels

Phytoplankton cultures were grown for a total of 40 days (and fed to the krill larvae for 32 days) in custom-made 45L transparent plastic growth bags (Entapack, Australia) in f/2 medium (Guillard and Ryther 1962; Guillard 1975), where nitrate, phosphate and silicate levels were adjusted to concentrations reported around O'Gorman Rocks, off Davis Station, Antarctica (Gibson 1998; Roden *et al.* 2013). We chose f/2 medium as it lacks any buffer that could affect the carbonate chemistry and trace metal speciation in the growth medium and would thereby affect medium pH and phytoplankton growth (Shi *et al.* 2009). Experimental conditions are listed in Table 3.1.

Algae were maintained in semi-continuous batch cultures by dilution every 2d with freshly prepared modified f/2 media of the respective CO_2 concentration. The culture bags were set up in a temperature controlled refrigerator averaging 3.5°C (range 2.4 - 4.2°C) and exposed to an incident irradiance of 142µmol m^{-2} s^{-1} (±9), on a 12:12h light:dark cycle. The control culture was bubbled with 0.2µm filtered ambient air and for the high CO_2 treatments,

ambient air (as above) used to bubble the cultures was enriched with pure CO_2 gas (BOC, Australia) via mass flow controllers (Horiba STEC SEC-E-40). Details on the CO_2 system are described elsewhere (Kawaguchi *et al.* 2011).

Table 3.1 Experimental conditions for phytoplankton cultures and krill larvae. Values are averages with standard deviations in brackets.

Species	**Acclimation [number of generations and days]**	**Light intensity [µmol m^{-2} s^{-1}] (±SD)**	**Average nutrient concentrations [µM] of fresh medium (±SD)**		
			NO_x (NO_3^- & NO_2^-)	**Si**	**P**
Pseudo-nitzschia subcurvata	5 - 9 generations, 7 days	142 (±9)	32 (±2)	70 (±0.9)	0.6 (±0.07)

	CO_2 concentration [µatm] (±SD) **pH**			
Pseudo-nitzschia subcurvata	326 (±30)	431 (±62)	712 (±62)	896 (±68)
	8.15 (±0.05)	8.01 (±0.05)	7.82 (±0.04)	7.72 (±0.04)
Krill	526 (±103)	482 (±81)	457 (±81)	479 (±63)
	8.09 (±0.08)	8.12 (±0.07)	8.15 (±0.07)	8.12 (±0.05)

Seawater pCO_2 concentrations were calculated with the CO2SYS.BAS Excel programme (Lewis and Wallace 1998), using the constants after Mehrbach et al. (1973) as refitted after Dickson and Millero (1987) and based on pH, total alkalinity, salinity, temperature and nutrient concentrations. Carbonate chemistry was monitored daily using pH measurements, taken with a Mettler Toledo Multi Seven pH meter (Mettler Toledo, Australia), calibrated to fresh tris- and aminopyridine artificial seawater buffer (Dickson *et al.* 2007), and alkalinity measurements, taken every 2d, prior to dilutions and poisoned with 25µl saturated mercuric chloride solution. Alkalinity samples were stored

refrigerated in the dark until analysis in a closed cell on a Total Alkalinity Titrator ATT-05 (Kimoto, Japan).

The phytoplankton cells were acclimated to the elevated CO_2 concentration for seven days, 5 - 9 generations, and then sampled for analysis of C:N, carbohydrate, fatty acid and lipid class content and composition. Cell density was measured daily to normalise these parameters to cell densities.

3.3.2. Phytoplankton physiological and biochemical analyses

Phytoplankton cultures were sampled in triplicate per treatment. Sufficient volume for all phytoplankton analyses listed below was harvested from the culture bags into sterile glass bottles and subsequently vacuum filtered for analyses as outlined below.

Phytoplankton growth rate and cell volume

Growth rates were determined six days a week via flow cytometry. Measurements were carried out using a BD FACSCalibur cytometer (Becton Dickson, USA) equipped with a 488nm argon laser. Cell dimensions were measured on Field Emission Scanning Electron Microscope images (JEOL JSM6701F, Japan). Cell volume of these lanceolate diatoms was approximated with the equation from Hillebrand et al. (1999).

C:N

100ml of culture were filtered onto precombusted (450°C overnight) 25mm Quartz filter. One quarter of each filter was used for analysis of C:N ratio via a Thermo Finnigan EA 1112 Elemental Analyser (CEInstruments, UK). Inorganic carbon was removed with 2M HCl prior to analysis.

Carbohydrates

The remaining portion of the C:N Quartz filter was used to determine particulate organic carbohydrates. Carbohydrates were first denatured into monosaccharides (adapted from Brown *et al.* 1998) and the carbohydrate concentration determined via the standard colorimetric method after Dubois et al. (1956) on a GBC UV-Vis 916 spectrophotometer (GBC Scientific Equipment, Australia).

Lipid extraction and analysis

For lipid analysis, 100ml of culture was vacuum filtered onto pre-extracted (chloroform : methanol 1:1 v/v) 25mm GF/F filters. Lipids were quantitatively extracted overnight with a modified one-phase (Bligh and Dyer 1959) method using water-methanol-chloroform (8:20:10 v/v/v). Phase separation occurred after addition of chloroform-water (10:10 v/v). Lipids were subsequently recovered in the lower solvent phase and this total solvent extract was concentrated via rotary evaporation at 40°C. Lipids were stored in glass vials in chloroform at -20°C until further analysis.

Lipid classes were quantified by analysis of an aliquot of the total solvent extract on an Iatroscan MK V TH10 thin-layer chromatography – flame

ionization detector (TLC-FID) (Iatron Laboratories, Tokyo, Japan) (Ackman 1981; Volkman and Nichols 1991). Samples were applied to silica gel SIII chromarods (5µm particle size) with 1µL micropipettes and placed in a glass tank lined with pre-extracted filter paper and hexane : diethyl ether : acetic acid solvent mix (60:17:0.1 v/v/v) for 20min to develop. This solvent mix resolves non-polar compounds such as wax ester (WE), triacylglycerol (TAG), free fatty acids and sterols. After development, the chromorods were oven dried at 100°C for 10min and analysed by an FID calibrated for each compound class (phosphatidylcholine, cholesterol, cholesteryl oleate, oleic acid, squalene, TAG (derived from fish oil), WE (derived from orange roughy, *Hoplostethus atlanticus*, oil; 0.1-10µg range). Peaks were quantified on an IBM compatible computer using DAPA Scientific software (Kalamunda, Australia). TLC-FID results are generally reproducible to ±10% of individual class abundances (Volkman and Nichols 1991).

The remaining total solvent extract was trans-methylated to produce fatty acid methyl esters (FAME) by heating in methanol : chloroform : concentrated hydrochloric acid (10:1:1 v/v/v) at 80°C for 2 hours (Christie 1982). FAME were extracted into hexane : chloroform (4 : 1 v/v) and concentrated under a stream of nitrogen. An internal injection standard (19:0 FAME) was added to each sample post trans-methylation and the fatty acid composition analysed by gas chromatography (GC) using an Agilent Technologies 7890A GC (Palo Alto, California, USA) fitted with an Equity – 1 fused silica capillary column (15m x 0.1mm i.d., 0.1µm film thickness), an FID, a split/splitless injector and

an Agilent Technologies 7683B Series auto sampler and injector. Helium was used as carrier gas.

Peaks were quantified with Agilent Technologies ChemStation software (Palo Alto, California, USA) and individual components were identified using mass spectral data and comparison of retention time data with those obtained for authentic and laboratory standards. GC – mass spectrometric analyses were performed on a Finnigan Thermoquest GCQ GC – mass spectrometer fitted with an on – column injector using Thermoquest Xcalibur software (Austin, Texas, USA) and a capillary column similar to that described above.

3.3.3. Feeding larvae phytoplankton grown at elevated pCO_2

Adult krill were caught in early 2009 off the Japanese station Syowa and maintained for experiments and breeding in the laboratory (Kawaguchi *et al.* 2010). Krill larvae were reared from eggs spawned from these adults in the laboratory. Once the larvae reached calyptopis I, they were fed a mixture of *Geminigera cryophila*, *Pyramimonas gelidicola*, and *Phaeodactylum tricornutum*, along with freshly hatched *Artemia* nauplii. Larvae of calyptopis I stage have high mortality rates (Ross *et al.* 1988). Once larvae reach calyptopis II, mortality rates drop (Ross *et al.* 1988), which is why the experiment was started with animals entering the calyptopis II stage. The krill feeding experiment lasted for 32 days. To minimize confounding effects caused by the variation in quality of larval egg batches, we pooled larvae from

several females for the experiment. Larvae hatched from several egg batches were picked randomly and distributed into 5L clear plastic containers filled with the phytoplankton culture of each CO_2 treatment. Each of the CO_2 treatments was replicated by three 5L containers with between 33 and 44 animals in each container. The 5L containers were floated in krill holding tanks maintained at 0.5°C.

The aim of this study was to isolate the effect of CO_2-altered algal nutritional quality on krill larvae. Prior to transferring krill larvae into the phytoplankton cultures, 10L cultures of each CO_2 treatment were bubbled with filtered ambient air that had been passed through a column filled with soda lime to absorb most of the CO_2. The pH of cultures subjected to elevated CO_2 was raised close to ambient ocean pH to avoid confounding pH effects on krill larvae by changing the water chemistry (Table 3.1). Phytoplankton cell density in the food provided to larvae was kept constant at around 21,000cells/mL across treatments, despite varying algal growth rates. Chilled 0.2µm filtered seawater was mixed with phytoplankton culture depending on the culture densities. Larvae were transferred into freshly prepared food every second day to minimise bacterial contamination and the possibility for phytoplankton cells from high CO_2 treatments to re-acclimate to ambient CO_2 concentrations in the krill jars and thereby confound the experiment. It was confirmed visually that food was always supplied in surplus, since uneaten food remained before the next feeding day. It was also observed that the digestive glands of larvae were green in colour throughout the experiment, indicating that phytoplankton cells were ingested continuously and therefore

larvae were not starved. Any food that settled to the bottom of the container was gently re-suspended with a pipette during daily checks.

The carbonate chemistry of the krill container was determined with the CO2SYS.BAS Excel programme via pH measurements along with alkalinity samples taken from the phytoplankton culture and the seawater used for mixing. The pH of the culture seawater mixture was measured prior to transferring larvae into the container and at the end of the two day period.

Moults and dead animals were collected as part of the daily inspection and preserved in 5% formalin-seawater.

3.3.4. Krill physiological and biochemical analyses

Krill larvae were transferred to 0.2µm filtered chilled seawater to wash off any phytoplankton culture. The larvae were then transferred onto filter, rinsed with 10mL 0.5M ammonium formate to remove any salt crystals and frozen in liquid nitrogen.

Larval growth

Larval growth over the course of the experiment was determined by measurement of telson length of moults shed at the end of the experiment. Since larvae were randomly attributed to the various treatments, the average body length at the start of the experiment is taken to be equal amongst treatments (confirmed by preliminary studies, unpublished data).

Moults were analysed with a Leica DFC 400 camera, mounted onto a Leica MZ 95 microscope (Leica), and associated image analysis software.

C:N

C:N composition was determined via a Thermo Finnigan EA 1112 Elemental Analyser (CEInstruments, UK). Krill larvae were freeze dried and weighed on a Sartorius SE2 Ultra Micro Balance (Sartorius, precision ± 0.0001mg) prior to analysis.

Lipid extraction and analysis

Lipid extraction and analysis of krill larvae on pre-extracted GF/F filter followed the same protocol as outlined above for phytoplankton cells on pre-extracted GF/F filter.

Amino acid composition

Krill amino acid composition was determined by Australian Proteome Analysis Facility, Sydney Australia. Freeze dried larvae were weighed and subjected to liquid hydrolysis in 6M HCl at 110°C for 24h. Amino acids were then derivatised using the Waters AccQTag Ultra chemistry and quantified in duplicate through ultra performance liquid chromatography. Cysteine, methionine and tryptophan were not analysed.

3.3.5. Statistical analysis

Phytoplankton

It was not practical to replicate culture bags for each CO_2 concentration, due to the large scale of the experimental bags. To get an estimate of sampling and analysis error, we chose to subsample each culture in triplicate per sampling date. However, these do not represent true replication, nor do repeated sampling dates. Bag to bag variations are therefore confounded with treatment effects. Statistical analysis and any conclusions drawn from these experiments have to be considered accordingly. Individual biochemical results in the phytoplankton samples were analysed with generalized additive models (GAM) (Wood 2006), smoothing over CO_2 concentrations, with separate splines per day. This procedure allowed for changes between the sampling days while keeping the focus on the trend in results with increasing CO_2 concentration. Differences in phytoplankton cell volume, length differences and growth rate were determined by analysis of variance (ANOVA) (Lefebvre *et al.* 2012). Statistical significance was accepted at $p \leq 0.05$.

Krill

Krill results were analysed with linear regression models, except for the mortality data. A quadratic log-hazard function was fitted to the daily mortality rates of krill fed *P. subcurvata*. This models the daily mortality rates as a function of time and CO_2 level. Since the log-hazard function is continuous, we integrated it by daily time steps to correspond to the data (Candy 1986). This means the striated appearance of the data points

corresponding to daily observations is represented by a smoothed curve over time for each CO_2 level treatment of the phytoplankton food. The usual exponential or Weibull survival time distributions have monotonically increasing log-hazard functions and cannot model the observed trend so we adopted a quadratic in time log-hazard function to model an initial delay in mortality, followed by high and then low mortality phases.

3.4. Results

3.4.1. Phytoplankton

Cell size and growth rate

After four weeks exposure to elevated CO_2 concentrations, post acclimation, *Pseudo-nitzschia subcurvata* showed no statistically significant difference in cell length, but cells exposed to 712µatm CO_2 had significantly lower cell volumes than cells exposed to 431µatm and 896µatm CO_2 ($p<0.05$, Table 3.2). Growth rate decreased significantly (between 34 and 45%) with increasing CO_2 concentration (Table 3.2).

Table 3.2 Post acclimation growth rates and cell volumes for *Pseudo-nitzschia subcurvata*. Standard deviation in brackets, n = number of samples. **p<0.01, * p < 0.05 significantly different from control group

pCO_2 [µatm]	326µatm	431µatm	712µatm	896µatm
Growth rate	0.66	0.40**	0.36**	0.43**
[d^{-1}]	(±0.19, n=11)	(±0.16, n=10)	(±0.11, n=10)	(±0.12, n=9)
Cell volume	133	142	121*	146.1
[μm^3]	(±45.1, n=69)	(±48.4, n=109)	(±46.8, n=105)	(±54.6, n=42)

Total lipid, lipid class and fatty acid concentrations

The GAM model did not detect a statistically significant trend in total lipid concentration per cell nor for lipid classes for *P. subcurvata* cultures (data not shown).

While total fatty acid content as well as SFA and MUFA content did not change with pCO_2 (Table 3.3), increasing CO_2 concentrations were accompanied by decreasing PUFA concentrations during the first half of the experiment (day 14 $p<0.005$ and 20 $p<0.05$, Fig. 3.1). The data from sampling days 27 and 34 indicate a recovery in PUFA concentrations in cultures exposed to elevated CO_2 concentration for longer periods of time.

DHA and EPA, the two main PUFA important for krill nutrition decreased with increased CO_2 concentration in *P. subcurvata* (Table 3.3), the trend was statistically significant on sampling days 20 ($p<0.005$) and 34 ($p<0.005$) for DHA and on days 14 ($p<0.05$) and 20 ($p<0.05$) for EPA. The ratio of SFA:PUFA increased on days 20 and 27 ($p<0.05$) in the highest CO_2 treatment (Table 3.3).

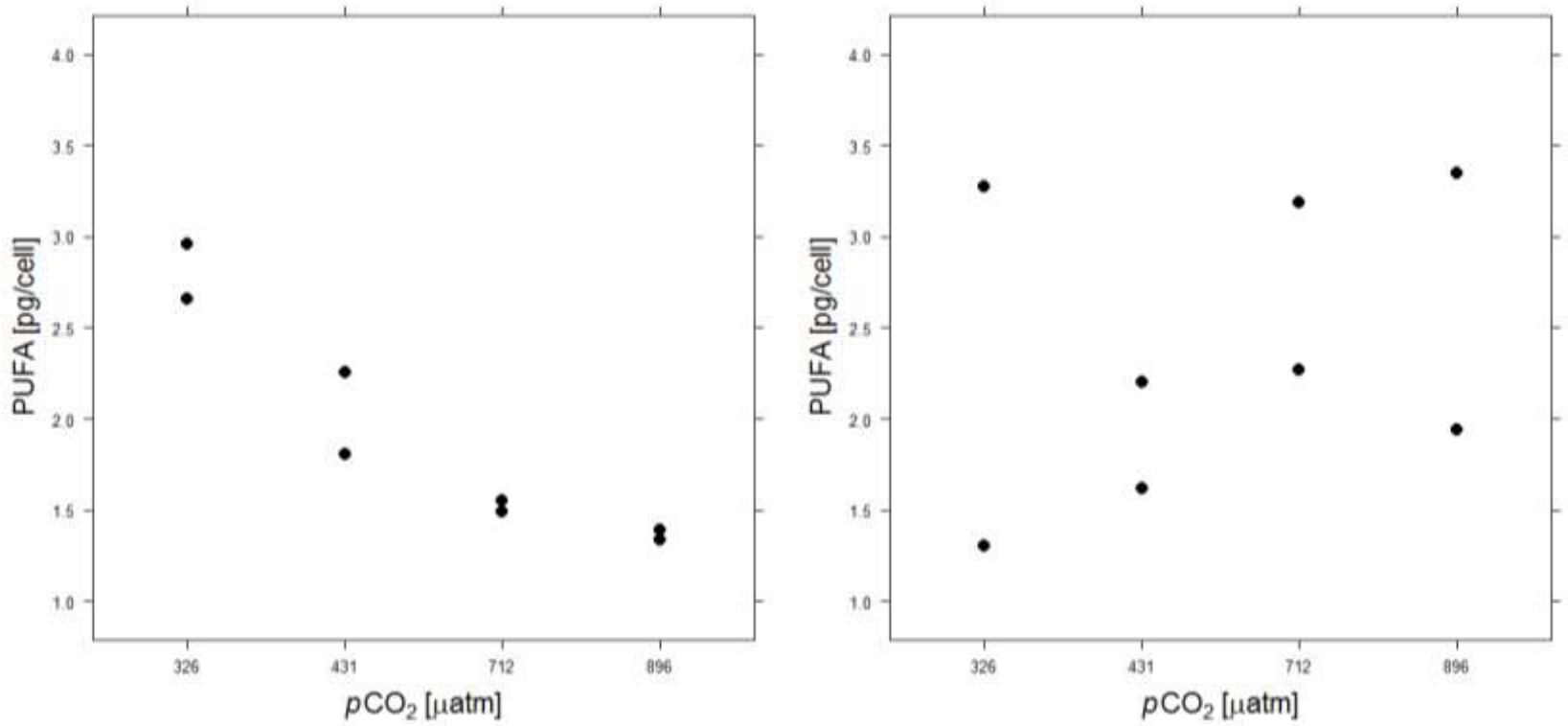

Figure 3.1 Polyunsaturated fatty acid (PUFA) content per cell [pg/cell] in *Pseudo-nitzschia subcurvata* at various CO_2 concentrations: during the first half of the experiment (left), days 14 ($p<0.005$) and 20 ($p<0.05$); during the second half of the experiment (right), days 27 and 34. Data points are averages of triplicate samples of the same culture.

Table 3.3 *Pseudo-nitzschia subcurvata* fatty acid content (fg/cell) under the four CO_2 concentrations (µatm). Numbers are averages over the whole experiment, SD = standard deviation, n = number of sampling dates.

Fatty acid	326µatm n = 4	431µatm n = 4	712µatm n = 4	896µatm n = 4
Total SFA	586 (±266)	525 (±210)	535 (±137)	628 (±114)
Total MUFA	1493 (±805)	1300 (±597)	1550 (±493)	1476 (±113)
Total PUFA	2547 (±866)	1970 (±308)	2126 (±791)	2006 (±936)
Total ω3	1669 (±647)	1341 (±232)	1469 (±625)	1291 (±795)
Total ω6	575 (±130)	420 (±67)	419 (±129)	493 (±115)
20:5ω3 (EPA)	1476 (±644)	1213.5 (±222)	1351 (±626)	1109 (±726)
22:6ω3 (DHA)	48 (±21)	31 (±12)	22 (±4)	31 (±5)
SFA : PUFA	0.23 (±0.04)	0.27 (0.10)	0.26 (±0.08)	0.35 (±0.13)
Total fatty acids	4626 (±1708)	3795 (±989)	4211 (±1238)	4110 (±1051)

Particulate organic carbohydrates and C:N

Cell-specific particulate organic carbohydrate (CHO) concentration increased at higher CO_2 concentration (Fig. 3.2). The GAM model showed a statistically significant trend at days 27 ($p<0.05$) and 34 ($p=0.05$).

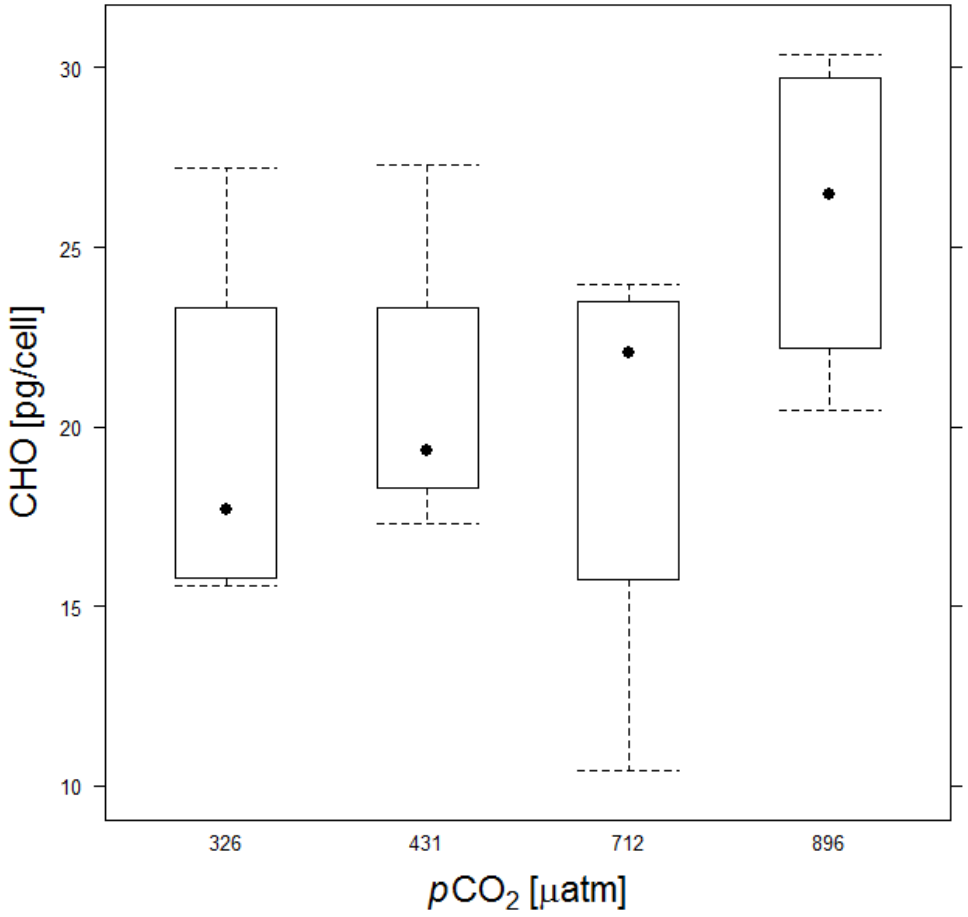

Figure 3.2 Carbohydrate (CHO) content per cell [pg/cell] in *Pseudo-nitzschia subcurvata* cultures. Data points are medians, boxes cover the lower and upper quartiles and the whiskers delineate the lowest and greatest values.

There was no clear trend in cell-specific carbon (C), nitrogen (N) (data not shown), percent carbon of dry mass (%C), percent nitrogen of dry mass (%N) or C : N in *P. subcurvata* cultures (Table 3.4).

Table 3.4 Elemental composition of *Pseudo-nitzschia subcurvata* in % dry mass. Results are averages over the whole experiment. Standard deviation in brackets and n = number of sampling dates.

Pseudo-nitzschia subcurvata	**326µatm n = 4**	**431µatm n = 4**	**712µatm n = 4**	**896µatm n = 3**
%C	4.3 (±1.4)	3.7 (±1.0)	3.7 (±0.6)	2.3 (±0.6)
%N	0.6 (±0.4)	0.7 (±0.6)	0.5 (±0.2)	0.6 (±0.6)
C:N	8.0 (±5.7)	7.7 (±3.5)	8.1 (±2.0)	6.6 (±3.8)

3.4.2. Krill

Krill larval development

There was no statistically significant difference between dry weight or telson length of larvae fed *Pseudo-nitzschia subcurvata*, irrespective of the pCO_2 at which these algae were grown (Table 3.5). Intermoult periods were similar for all treatments and ranged between 10 and 12 days.

Table 3.5 Dry weight [mg], telson length [mm] and intermoult period [d] of krill larvae fed *Pseudo-nitzschia subcurvata* cells grown at CO_2 concentrations of 326µatm, 431µatm, 712µatm and 896µatm. Standard deviation in brackets.

	326µatm	**431µatm**	**712µatm**	**896µatm**
Dry weight [mg]	0.33 (±0.09)	0.35 (±0.10)	0.31 (±0.08)	0.33 (±0.12)
Telson length [mm]	0.85 (±0.06)	0.85 (±0.08)	0.86 (±0.05)	0.85 (±0.06)
Intermoult period [d]	10 (±2)	10 (±1)	10 (±1)	10 (±2)

Total lipid, lipid class and fatty acid concentrations

No significant differences were found in total lipid per dry weight or the proportional composition of lipid classes in larvae fed *P. subcurvata* cultures acclimated to different CO_2 concentrations (Table 3.6).

Table 3.6 Lipid class composition (as percent of total lipid) in krill larvae fed *Pseudo-nitzschia subcurvata* cells grown at CO_2 concentrations of 326µatm, 431µatm, 712µatm and 896µatm. Standard deviation in brackets, n = 3, each sample contained between 6 and 12 larvae

	326µatm	**431µatm**	**712µatm**	**896µatm**
%TAG	27.5 (±3.6)	30.6 (±12.9)	38.7 (±3.2)	30.3 (±4.8)
%PL	67.1 (±5.3)	63.2 (±13.2)	55.9 (±3.2)	63.6 (±4.4)
Total lipid content per dry weight [µg/mg]	113.2 (±11.7)	131.0 (±26.0)	132.4 (±25.5)	130.6 (±25.7)

The DHA : EPA ratio in krill larvae generally decreased with increasing CO_2 concentration of the food medium, and was lowest in larvae fed algae grown at the second highest CO_2 concentration, 712µatm (Table 3.7). Concentrations of monounsaturated fatty acids (MUFA) and ω6 PUFA increased with increasing CO_2 concentration of the algal growth medium. As seen in the changes in the DHA : EPA ratio, the largest increase in MUFA concentrations occurred in larvae fed algae grown at the second highest CO_2 concentration, 712µatm (Table 3.7).

Table 3.7 Fatty acid content in µg per mg dry mass and the DHA:EPA ratio for krill larvae fed *Pseudo-nitzschia subcurvata* cells grown at CO_2 concentrations of 326, 431, 712 and 896µatm. Standard error in brackets and n = number of samples, with each sample containing 2-3 larvae. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$ significantly different from control group.

Fatty acid [µg/mg]	**326µatm n = 3**	**431µatm n = 3**	**712µatm n = 3**	**896µatm n = 2**
MUFA	26.45 (±4.22)	33.91 (±4.55) p = 0.05	35.64 (±3.85)*	33.22 (±1.32)
PUFA	42.93 (±6.15)	49.40 (±10.31)	48.50 (±5.07)	46.56 (±1.85)
ω3	36.40 (±5.20)	41.79 (±8.92)	40.07 (±3.84)	38.42 (±1.92)
ω6	3.94 (±0.43)	3.58 (±0.23)	4.37 (±0.31)	5.01 (±0.10)*
DHA : EPA	0.049 (±0.001)	0.042 (±0.006)*	0.037 (±0.001)**	0.042 (±0.001)

Elemental composition

Although there was no clear trend in the elemental composition of the diatom itself, krill larvae fed the highest pCO_2-grown diatoms had slightly increased %C and C : N (Table 3.8). Percent sulphur of dry mass (%S) could not be measured in the phytoplankton samples, however, the %S of krill larvae decreased significantly as the concentrations of CO_2 at which the alga was grown increased (Fig. 3.3).

Table 3.8 Elemental composition of krill larvae fed *Pseudo-nitzschia subcurvata* cells grown at CO_2 concentrations of 326µatm, 431µatm, 712µatm and 896µatm in % dry mass. Krill results are from whole animals at the end of the experiment. Standard deviation in brackets and n = number of samples, with each krill sample containing between 2 and 3 larvae.
* p < 0.05, ** p < 0.005, *** p < 0.001 significantly different from control group

Krill fed *Pseudo-nitzschia subcurvata*	**326µatm n = 9**	**431µatm n = 9**	**712µatm n = 9**	**896µatm n = 8**
%C	39.6 (±2.3)	38.9 (±2.3)	38.8 (±2.0)	43.0 (±4.9)*
%N	10.3 (±0.9)	10.2 (±0.7)	10.0 (±0.9)	9.6 (±1.0)
%S	0.8 (±0.1)	0.8 (±0.1)	0.7 (±0.1) *	0.6(±0.1)***
C:N	3.9 (±0.5)	3.8 (±0.4)	3.9 (±0.4)	4.5 (±1.0)*

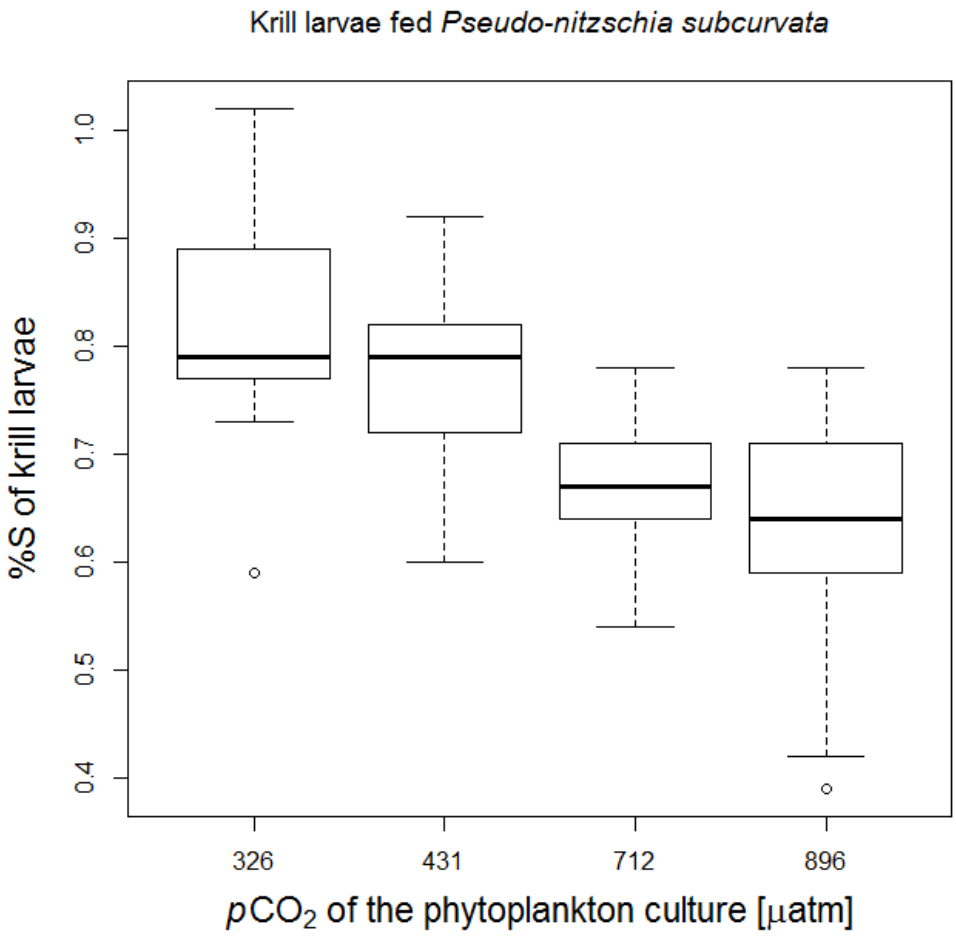

Figure 3.3 Sulphur content (% dry mass) in krill larvae fed *Pseudo-nitzschia subcurvata* grown at various pCO_2.

Krill amino acid composition

There was no trend in the concentrations of total bound amino acids / mg dry weight and the overall amino acid profile in krill larvae feeding on *Pseudo-nitzschia subcurvata* cultures (data not shown).

Mortality

Figure 4 depicts daily mortality rates calculated as the ratio of the sum of dead larvae to the sum of live larvae for all three replicates per treatment. The thick black line represents the modelled daily mortality rates together with twice the standard error of the difference as upper and lower confidence bounds (thin black lines) to determine statistical significance at an approximate 95% level of probability. Of all larvae fed *P. subcurvata* grown at 896µatm CO_2, 48% died during the course of the experiment compared to 30% in the control group (39% in larvae fed algae grown at 431µatm and 712µatm CO_2). Daily mortality rates increased in all four treatments from the start of the experiment until approximately day 15, after which daily mortality rates declined again in all four treatments. The highest daily mortality rate occurred in larvae fed *P. subcurvata* grown at 896µatm CO_2. Daily larval mortality also started to increase more sharply in larvae fed *P. subcurvata* grown at 896µatm CO_2 compared to larvae in the control treatment ($p<0.05$) (Fig. 3.4). The highest daily mortality rates occurred during calyptopis II stage in all four treatments.

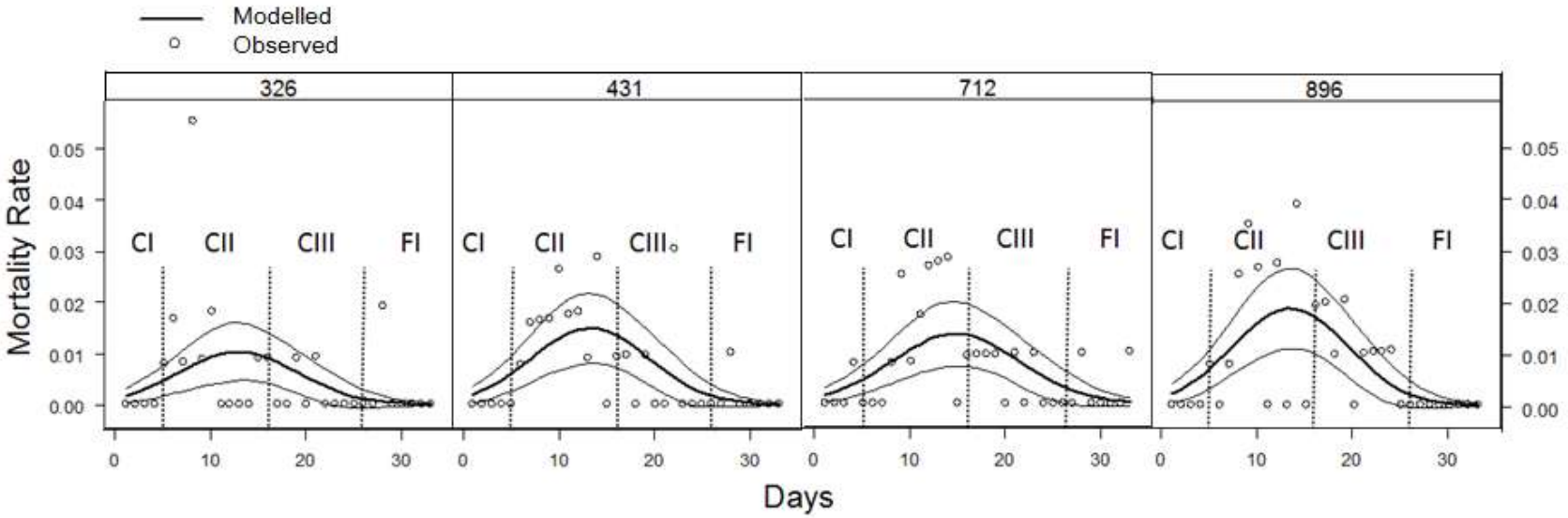

Figure 3.4 Daily mortality rates for krill larvae fed *Pseudo-nitzschia subcurvata* cells grown at 326, 431, 712 and 896µatm CO_2. Circles represent the ratio of the sum of dead larvae to the sum of live larvae for all three replicates per treatment; the thick black line represents the modelled daily mortality rates while the black lines represent upper and lower confidence bounds (twice the standard error of the difference) to determine statistical significance at an approximate 95% level of probability. Dashed vertical lines indicate the approximate timing of metamorphoses between larval stages.

3.5. Discussion

Phytoplankton

Changes we observed in nutritional quality of *Pseudo-nitzschia subcurvata* were similar to those reported elsewhere and affected survival of the larvae. PUFA concentrations of cultures in the highest CO_2 treatment decreased during the first half of the experiment by about 50% compared to the control culture. This is a larger decrease than has been reported by Rossoll *et al.* (36% at 761µatm) in the diatom *Thalassiosira pseudonana* (2012). Detailed studies of the mechanisms and pathways of lipid and fatty acid production in other organisms suggest that external and internal pH influence lipid and fatty acid production. A decrease in external pH can translate into a decrease in internal pH (Lane and Burris 1981). Decreased internal pH in turn was reported to suppress phospholipid metabolic genes in yeasts (Young *et al.* 2010), and a higher unsaturation of fatty acids at ambient CO_2 concentrations compared to CO_2-enriched cultures of *Chlorella kessleri* was at least partially attributed to suppressed fatty acid synthesis and thus the promotion of desaturation of pre-existing fatty acids (Sato *et al.* 2003). A higher degree of membrane lipid fatty acid saturation could be a mechanism to maintain internal pH, since a higher degree of fatty acid saturation leads to less fluid and more CO_2-permeable cell membranes (Rossoll *et al.* 2012).

P. subcurvata showed a 32% increase in carbohydrate concentrations at the highest CO_2 concentration compared to the control culture. This is similar to

findings in *Phaeocystis antarctica* cultures at 993µatm (average pH 7.67) (30% increase) (Wynn-Edwards *et al.*, submitted). However, this is a greater increase than the 23% increase in cellular content of glucan, a storage carbohydrate, reported in the marine diatom *Skeletonema costatum* grown at pH 7.5 (Taraldsvik and Myklestad 2000). In the latter experiment the pH of the growth medium was manipulated via addition of acid / base in contrast to CO_2 gas as used here, which might explain the difference in results. The increased storage of carbohydrate in *P. subcurvata* may be due to increased availability of carbon compared to other elements, such as nitrogen and phosphorus, which were supplied at low concentrations.

The C:N ratio of autotrophs is an important characteristic of their nutritional quality for consumers. C:N ratios much higher than that of the consumer can lead to low grazer growth efficiencies per dietary C (Vrede *et al.* 2004). We did not observe significant changes in C:N ratio of *P. subcurvata*. However it is possible that changes in the biochemistry of this alga were in part masked by diurnal cycles, despite our efforts to obtain samples at the same time of day in the 12:12h light : dark cycle. Previous studies have shown that diurnal variations in C:N:P were of similar magnitude as CO_2–induced differences on C:N:P (Burkhardt *et al.* 1999).

We used low nutrient concentrations approximating those reported for the area where our phytoplankton strains were isolated. This is in contrast to other studies which used high nutrient enriched culture media such as f/2 (Burkhardt *et al.* 1999; Berge *et al.* 2010). Gervais *et al*. (2001) did not detect

any effect of CO_2 concentration on algal elemental composition under phosphorus limitation. This could explain why we found very little evidence for CO_2-induced changes in elemental composition of the diatom at the low nutrient concentrations used here.

Growth rates decreased between 34 and 45% with increased CO_2 concentration. This is in contrast to other studies in which CO_2 stimulated growth rate (Riebesell *et al.* 1993; Schippers *et al.* 2004; Kim *et al.* 2006; Wu *et al.* 2010), but agrees with findings by Mueller *et al*. (2010) in long-term experiments with the coccolithophores *Emiliania huxleyi* and *Coccolithus braarudii*. Decreased availability of food due to slowed growth rates could be detrimental to krill larvae, especially during their first winter. Small changes in growth rate in a single-species laboratory experiment might not be viewed as ecologically significant. However, in a natural mixed community and in the presence of others stressors, such small changes to the physiology of a species could provide enough competitive disadvantages to decrease the abundance of the affected species.

Krill biochemical composition

While %C and C:N were significantly increased in larvae fed *P. subcurvata* grown at the highest CO_2 treatment, the strongest trend in larval biochemistry was found to be a significant decrease in %S of dry mass in larvae fed *P. subcurvata* grown at elevated CO_2 concentration. If phytoplankton are phosphorus limited they can replace phospholipids with sulphur containing membrane lipids (van Mooy *et al.* 2009). Phosphorus concentration in the

base seawater, used to make up the culture medium, was much lower than expected. Nutrient concentration results of the base seawater were not available during the experiment and thus we did not increase the phosphorus addition to compensate for low background levels. Control cultures which grew faster possibly became phosphorus limited sooner than slower growing high – CO_2 cultures. Hence control cultures could have incorporated more sulphur containing membrane lipids than high – CO_2 cultures, which would have become less phosphorus limited due to their reduced growth rates and thus would have incorporated less sulphur instead of phosphorus in their membrane lipids. This could explain the significant decrease in %S of dry mass in krill larvae that fed *P. subcurvata* grown at elevated CO_2 concentration.

The significant decrease in %S in larvae fed high – CO_2 grown algae may also be the result of decreased levels of sulphur – containing components such as dimethylsulfoniopropionate in the phytoplankton at increased CO_2 concentration, although no analyses were performed. Hopkins *et al.* (2009) and Arnold *et al.* (2013) reported a decrease in the related compound, dimethyl sulfide, at elevated CO_2 concentration, whereas Wingenter (2007) observed the opposite. The species composition of these two experiments was different and it is possible the opposing results can be explained by different species-specific responses to increased CO_2 availability.

Krill mortality

Our experiment showed that larval krill mortality can be influenced by diet grown at elevated pCO_2. In the case of larvae feeding on *P. subcurvata*, daily mortality rates had the shape of a bell curve for all treatments, suggesting that there could be a proportion of the population that is more susceptible to stressors and consequently has lower survival rates. The susceptibility of these larvae appeared to be exacerbated by diet. We propose that this more susceptible proportion died off first, leaving the more resilient animals. This would explain the initial increase in daily mortality rate, followed by a decrease once most of the more susceptible animals had died. An alternative explanation would be that there is a developmental stage specific mortality. Dashed vertical lines in Fig. 3.4 indicate approximate timings of the metamorphoses between larval stages. The highest larval stage specific mortality occurred during the calyptopis II stage and decreased once larvae reached calyptopis III. Daily rates of krill mortality increased with increasing CO_2 concentration of the algal growth medium. The decrease in essential PUFA, in particular DHA and EPA, in *P. subcurvata* cultures of elevated pCO_2 in the first half of the experiment and the increase in the ratio of SFA : PUFA and particulate carbohydrates are consistent with the trend in krill mortality in the high CO_2-food treatments. However, these changes in the food source were not directly reflected in the biochemistry of the krill. This could be due to the fact that only survivors were collected and analysed at the end of the experiment. These animals were possibly better adapted to nutritionally inferior food, which is why they survived. Equally the amino acid profile of survivors did not show any changes with food source, either

because it is a highly conserved trait or because we only analysed surviving larvae. Analysis of dead larvae is unreliable since it is impossible to preserve them at the very moment of death, despite frequent checks. Partial decay and / or cannibalism would also compromise results for the analysis of dead larvae.

To exclude confounding effects of low pH seawater on krill larvae, phytoplankton cultures were bubbled with CO_2-poor air to lower the pCO_2 levels to ambient before transferring the larvae into the cultures. Fresh culture mixes were prepared every 2nd day. It is unclear what effect this rapid change in CO_2 concentration environment had on the phytoplankton physiology and biochemistry. Subsequent observations with juvenile krill showed that up to 80% of the cells are filtered out of the water by the krill in the first 7 hours (data not shown). It is therefore likely that at the time the phytoplankton cells were consumed by the krill, the time of exposure to a CO_2 concentration different from what they had been acclimated to was only minimal and possible changes to their nutritional quality were likely to be minor. Rossoll *et al*. (2012) reported changes in PUFA profiles in the diatom *Thalassiosira pseudonana* within 10h of changing the CO_2 concentration. Due to the uncertainty of rapid changes in the carbon chemistry on phytoplankton biochemistry, we suggest minimizing exposure times of phytoplankton cells to altered CO_2 concentrations before they are either grazed by the krill or removed from the experimental unit. This could be facilitated by means of a flow-through system where food supply to the krill is continuous and thus phytoplankton cells are constantly flushed out and renewed or grazed by the krill.

A reduction in egg production rates, growth and larval survival due to a deterioration in the nutritional quality of phytoplankton has been reported for several algae and grazer species (Urabe and Sterner 1996; Urabe *et al.* 2002; Urabe *et al.* 2003; Diekmann *et al.* 2009; Rossoll *et al.* 2012). However, species-specific differences in the phytoplankton response (Burkhardt and Riebesell 1997; Burkhardt *et al.* 1999) and most likely also the susceptibility of the grazer, make it difficult to extrapolate single-species experiments to whole ecosystem food webs.

A further complication arises from the fact that grazers usually have a number of phytoplankton species available as food and some studies suggest that krill can selectively choose food particles of superior nutritional quality (Haberman *et al.* 2003). Thus deterioration in nutritional quality of one phytoplankton species could be compensated for by selective grazing. Such a scenario raises the question how the whole phytoplankton community will change under future ocean acidification scenarios. A recent study with a mixed community concluded that increased CO_2 concentration had no effect on overall planktonic food quality, although they found a negative correlation between pCO_2 and relative amounts of EPA (Leu *et al.* 2013). The nutritional quality of a plankton community under ocean acidification will depend on if and how individual species are affected by increased CO_2 concentrations, how the species composition will change and what the nutritional value of the new dominant species will be.

Based on the recent findings on the direct and indirect effects of ocean acidification on Antarctic krill, it is difficult to estimate the relative importance of either of these impacts, ocean acidification and altered nutritional quality of phytoplankton. Kawaguchi *et al*. (2013) found strong direct effects of increased seawater pCO_2 on hatch rates; an elevation of seawater pCO_2 to 1750µatm reduced mean hatch rates to 20% of control levels. A change in the biochemistry of *P. subcurvata* increased larval mortality rates from 30% to 48% in our study. This is a moderate increase in larval mortality compared to the reduction in hatch rates due to ocean acidification alone, however, a small increase in larval mortality in the laboratory can have much larger impacts in the natural population where other stressors might exacerbate the negative effect. Findings by Yoshida *et al.* (2011) reported a reduction in hatching success from 24.3% to 7% in females who were fed a diet insufficient (based on fatty acid profiles). These findings suggest that krill embryonic development is strongly influenced by the diet of spawning females.

3.6. Conclusion

In conclusion, our results suggest that CO_2-induced effects on phytoplankton can be transferred to the next trophic level and this is in agreement with similar studies. However, our experiment could not conclusively explain why feeding on high-CO_2 grown phytoplankton resulted in increased larval mortality and more phytoplankton species should be tested for their response to increasing levels of ocean acidification. To improve our ability to

extrapolate laboratory based results into the field, mixed phytoplankton species experiments with Antarctic krill larvae and adults as well as combined experiments which investigate the impact of ocean acidification and altered phytoplankton biochemistry together are recommended.

4. Development of a continuous phytoplankton culture system for ocean acidification experiments

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4.1. Abstract

Around one third of all anthropogenic CO_2 emissions have been absorbed by the oceans, causing changes in seawater pH and carbonate chemistry. These changes have the potential to affect phytoplankton, which are critically important for marine food webs and the global carbon cycle. However, our current knowledge of how phytoplankton will respond to these changes is limited to a few laboratory and mesocosm experiments. Long-term experiments are needed to determine the vulnerability of phytoplankton to enhanced pCO_2. Maintaining phytoplankton cultures in exponential growth for extended periods of time is logistically difficult and labour intensive. Here we describe a continuous culture system that greatly reduces the time required to maintain phytoplankton cultures, and minimises variation in experimental pCO_2 treatments over time. This system is simple, relatively cheap, flexible, and allows long-term experiments to be performed to further our understanding of chronic responses and adaptation by phytoplankton species to future ocean acidification.

4.2. Introduction

A human-induced increase in atmospheric pCO_2 is changing the carbonate chemistry of the oceans. Termed "ocean acidification", these changes may threaten a range of marine organisms (Spero *et al.* 1997; Bijma *et al.* 1999; Kleypas *et al.* 1999; Marubini and Atkinson 1999; Riebesell *et al.* 2000; Paulino *et al.* 2008). To better understand and predict the impact of ocean acidification on marine organisms, experimental research must simulate natural changes in ocean chemistry.

A range of methods have been applied to manipulate seawater pH and CO_2 concentrations in ocean acidification experiments, with different effects on the carbonate chemistry (Hurd and Hepburn 2009; Shi *et al.* 2009). This has made it difficult to compare results (Riebesell *et al.* 2000; Iglesias-Rodriguez *et al.* 2008; Rost *et al.* 2008) and therefore this issue has been addressed by the publication of a number of best practice guides for ocean acidification research (Kleypas *et al.* 2006; Dickson *et al.* 2007; Gattuso and Lavigne 2009; Riebesell *et al.* 2010). There is no consensus on the need or duration of acclimation periods prior to experiments (Riebesell *et al.* 2010), however, it is now common practice to acclimate cells for about 7–9 generations (Riebesell *et al.* 2000; Berge *et al.* 2010) before any measurements are taken. Furthermore, medium- to long-term ocean acidification experiments performed over many generations are recommended to assess natural plasticity (Riebesell *et al.* 2010).

In phytoplankton experiments on the effects of perturbations, such as altered seawater carbonate chemistry, algae are commonly maintained in exponential growth phase, so that changes among treatments are not masked by differences due to growth stage (Thornton 2009; Boelen *et al.* 2011; Arnold *et al.* 2013). Maintaining constant cell physiology also translates into consistent rates of CO_2 draw-down, which makes it easier to maintain stable carbonate chemistry for the duration of the experiment.

There are two ways to maintain phytoplankton in exponential growth, either semi-continuous or continuous cultures. Semi-continuous culturing requires dilution of the exponentially growing culture with fresh medium at regular intervals. The frequent culture dilutions can be time consuming and labour intensive, particularly for long-term experiments. Depending on how often the dilutions are performed, periodic variations in nutrient concentrations can also affect the physiological state of the cells (Holland *et al.* 2004; Bonachela *et al.* 2011). For ocean acidification experiments with phytoplankton, reduction of culture medium pCO_2 by photosynthesis is of particular concern and this is affected by cell density. Preliminary experiments by the authors showed that an exponentially growing semi-continuous diatom culture decreased the average pCO_2 to around 159 µatm despite being continuously bubbled with 390 ppm ambient air (data not shown).

The problems commonly associated with semi-continuous culturing can be minimised by use of a continuous system, which provides constant dilution of cultures and supplies fresh nutrients, thereby avoiding episodic changes in the

cell physiology. Through the continuous influx of pCO_2-adjusted media, carbonate chemistry is stabilized even at higher cell densities. Furthermore, the portion of the culture that is replaced by fresh media can be collected for analysis, thereby circumventing the issues arising from episodic large removal of cells such as during semi-continuous culturing. The use of an automated system can also reduce the time and effort needed to maintain experimental conditions. Therefore our aim was to design a simple, flexible and comparatively cheap continuous culture system for use in ocean acidification experiments that automatically supplies fresh media at the target pCO_2 with a pCO_2 stability comparable to that of other long-term experiments. Our experiments used three Antarctic phytoplankton species namely, the prasinophyte *Pyramimonas gelidicola,* the haptophyte *Phaeocystis antarctica* and the diatom *Fragilariopsis cylindrus*.

4.3. Materials and Methods

4.3.1. Experimental design and materials

Our continuous culture system was used to grow phytoplankton species with triplicate culture bags for each of three CO_2-enriched treatments and one control treatment supplied with ambient air. Dilution rates of the bags were controlled by varying the effective culture volume within each bag, rather than the flow rate of nutrient addition to each bag. This allowed use of two 12-channel peristaltic pumps rather than 24 single-channel pumps that would otherwise be required. The two 12-channel pumps (Masterflex, John Morris Scientific Pty Ltd, Chatswood, NSW, Australia) supplied a constant flow of fresh culture medium to 24 phytoplankton cultures (in this case with two

separate species, 12 bags per species, 3 bags per CO_2 treatment) to maintain the cells in exponential growth (Fig 4.1). Each culture bag was inoculated with a clonal culture of phytoplankton and randomly attributed to the treatments and subsequently the peristaltic pumps delivered a total volume of 1.8 L of medium to the cultures. Initial cell densities for each species were: *Fragilariopsis cylindrus* ~2400 cells/mL, *Pyramimonas gelidicola* ~8000 cells/mL and *Phaeocystis antarctica* ~ 9500 cells/mL. We chose f/2 medium (Guillard and Ryther 1962; Guillard 1975) as it lacks any buffer that could affect the carbonate chemistry and trace metal speciation in the growth medium, which would thereby affect medium pH and phytoplankton growth (Shi *et al.* 2009). Nitrate and phosphate were adjusted to concentrations reported where these species were isolated from (near-shore coastal waters around O'Gorman Rocks, off Davis Station Antarctica (Gibson 1998; Roden *et al.* 2013). Silicate concentrations were lower than around O'Gorman Rocks but not limiting (Harrison *et al.* 1977; Egge and Aksnes 1992). Once the species had reached exponential growth, the peristaltic pumps were set to a rate that maintained exponential growth at a constant cell density.

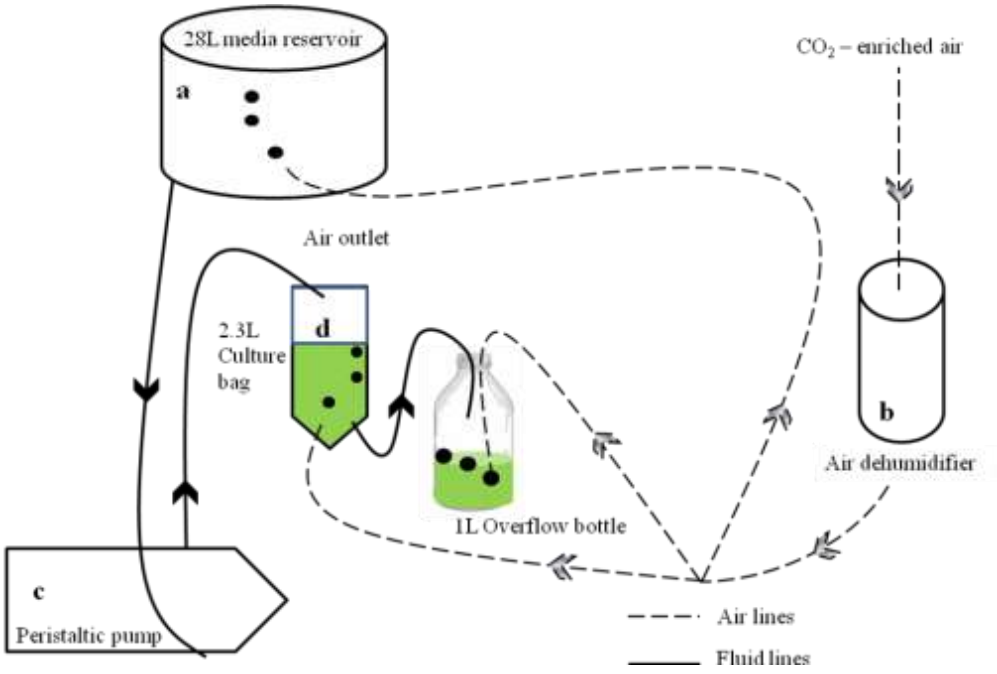

Figure 4.1 Overview schematic diagram of the ocean acidification continuous system. For simplicity only one CO_2 treatment and culture bag is shown (a) Eight 28 L media reservoirs supplied the cultures with media of the respective pCO_2. The reservoirs were continuously bubbled with CO_2-enriched air of the respective concentration; (b) Air/CO_2-mix bubbling through the reservoirs, all culture bags and overflow bottles was first dehumidified by passing through a silica-gel filled cylinder with an activated charcoal stage at the end to remove any organic contaminants; (c) Peristaltic pumps delivered the media from the reservoirs to each culture bag at a rate equivalent to the growth rate of the culture; (d) Culture bags and overflow bottles were continuously bubbled to maintain stable carbonate chemistry. Dilution of culture with fresh medium caused the culture to overflow into a sterile 1 L bottle, the content of which was used for subsequent analyses. Further details of the overflow bottle arrangements are depicted in Figure 4.2 (Wynn-Edwards *et al.* 2014a).

For a constant supply of fresh medium there were eight media reservoirs, two for each CO_2 treatment. The reservoirs were made out of transparent, sterile, polyethylene plastic culture growth bags (Entapack Pty Ltd, Dandenong, VIC, Australia). The peristaltic pumps were connected to the media reservoirs via manifolds that allowed the switch from one reservoir to the other once one was depleted. The empty reservoirs were filled with filter sterilized media and bubbled with CO_2-enriched air of the respective pCO_2. Culture bags were custom-made from the same material (Entapack Pty Ltd, Dandenong, VIC, Australia), heat-sealed to a prescribed pattern and held up to 2.3 L. The

culture bags tapered towards the base where the CO_2-air inlet was positioned (Figure 4.2). The conical shape of the culture bag and the position of the air inlet ensured mixing of the entire bag contents and minimized cells settling out of the water column. The bags were hung from a metal frame in three rows of eight.

We used 2.06 mm internal diameter (ID, silicone tubing (Masterflex, John Morris Scientific Pty Ltd, Chatswood, NSW, Australia)) for media transport and 5 cm long Teflon tips (2.13 mm ID) to puncture the culture bags for media supply. Dilution of each bag caused the culture to overflow via 2.13 mm ID Teflon tube connected to large silicone tubing (2.57 mm ID) into a sterile 1 L glass overflow bottle (Fig 4.2). Each overflow bottle was located next to the culture and flushed with CO_2 mixture to ensure the same treatment conditions (light, temperature and CO_2) as the experimental culture. The contents of the overflow bottle were then sampled for experimental analyses.

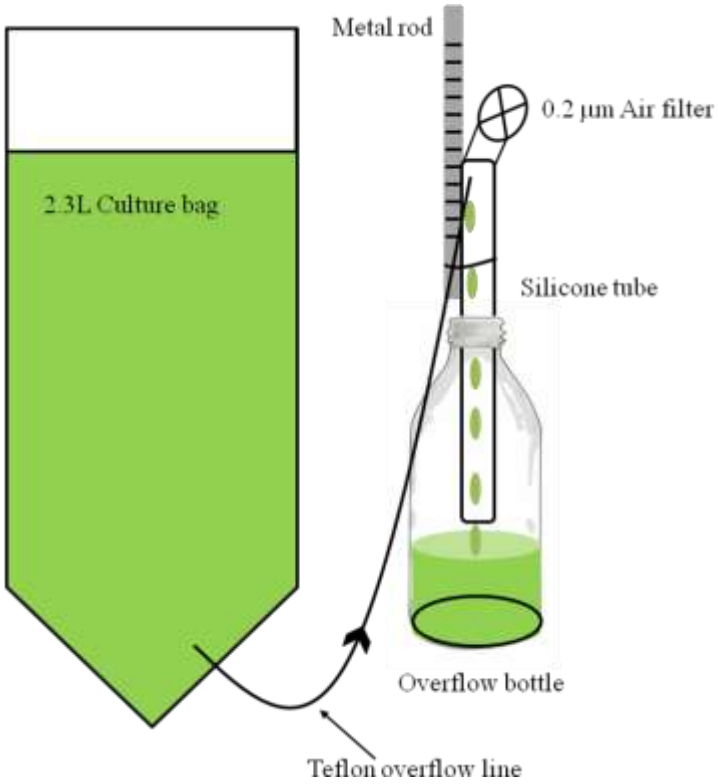

Figure 4.2 Schematic diagram of a culture bag and its attached overflow bottle. As the peristaltic pump added media to the bag, excess culture flowed through the 1 mm Teflon overflow line into a silicone tube and dripped from there into the overflow bottle. The height of the top of the Teflon overflow line relative to the culture bag controlled the flow rate of culture out of the bag: the lower the tip of the Teflon line compared to the culture bag, the greater the flow rate. The position of the Teflon overflow line was adjusted by moving it up and down a metal rod, fastened next to each overflow bottle. Individual adjustment of the outflow relative to the constant rate of influx from the peristaltic pump (Fig 4.1) could be used to determine the overall volume of each culture bag. The silicone tube was open to the outside pressure via a 0.2 µm air filter to prevent the culture from siphoning out of the culture bag. For simplicity air lines and air outlets are not included in this diagram.

Teflon tubing was used for the overflow line as it is chemically inert, can be autoclaved and is very smooth, thereby avoiding settlement and/or adhesion of cells to the tube wall. The overflow silicone tubing was open to outside pressure by means of a 0.2 µm air-filter on top. This ensured excess culture gently overflowed into the bottles rather than establishing a siphon.

The dilution rate of each culture was adjusted by changing the culture volume of each bag rather than the flow rate, since the multichannel pumps delivered the same inflow of media to each bag. The position of the Teflon overflow line in relation to the culture bag controlled the rate of culture flowing out of the bag gravimetrically. The lower the tip of the Teflon line compared to the culture bag the more culture overflowed. The rate of culture overflowing determined the overall volume of each culture bag. Adjustment of the culture volume in this way, while influx of media was constant, was a flexible method to adjust dilution rates individually for each culture bag. Thus cell abundance could be maintained in each bag, despite any differences in growth rate among species and CO_2 treatments. The position of the Teflon overflow line was adjusted by moving it up and down a metal rod, fastened next to each overflow bottle.

4.3.1.1. Experimental conditions

The cultures, overflow bottles and media reservoirs were kept in a temperature controlled refrigerator, maintained at an average 2.9 °C. The culture bags were positioned in front of fluorescent lights with irradiance of 267 ± 6.9 $\mu mol \cdot m^{-2} \cdot s^{-1}$, approximating the light intensities at 5 m water depth around Davis Station, East Antarctica (Thomson *et al.* 2008). Each culture and overflow bottle was continuously bubbled with ~270 mL/min CO_2–regulated air to achieve the target pCO_2 concentrations for the experimental treatments. On the basis of an average atmospheric pCO_2 of 390 ppm and constant air flow rates, the required addition of food grade CO_2 gas (BOC, Hobart, TAS,

Australia) was calculated and added with mass flow controllers (Horiba STEC SEC-E-40). CO_2 could also be added to control cultures to compensate for photosynthetically-driven CO_2 draw-down in dense cultures. The CO_2-enriched air passed through silica gel to absorb moisture and reduce condensation in air lines once it entered the refrigerator. Activated charcoal removed any organic contaminants, and 0.2 µm filters at the entry to each culture, overflow bottle and media reservoir assured sterility. To maintain constant pCO_2, the medium supplied to each culture was continuously bubbled with CO_2-enriched air of the respective concentration.

4.3.1.2. Carbonate chemistry

To monitor carbonate chemistry daily in the culture bags, pH was measured with a Mettler Toledo Multi Seven pH meter (Mettler-Toledo Ltd., Port Melbourne, VIC, Australia), calibrated to fresh Tris- and Aminopyridine artificial seawater buffer, made according to the SOP 6a in "Guide to best practices for ocean CO_2 measurements" (Dickson *et al.* 2007). Alkalinity samples (50 mL) were taken at regular intervals, poisoned with 25 µL saturated mercuric chloride solution and stored refrigerated in the dark until analysis in a closed cell on a Total Alkalinity Titrator ATT-05 (Kimoto, Osaka, Japan). A temperature probe logged the air temperature inside the refrigerator every 30 min. CO_2 concentrations were calculated with the CO2SYS.BAS Excel programme (Lewis and Wallace 1998) based on total alkalinity, pH (seawater scale), temperature (average of 2.9 ± 0.5 °C) and nutrient concentrations using the constants after Mehrbach *et al.* (1973) as refitted after Dickson and Millero (1987).

4.4. Results and Discussion

4.4.1. Stability of the carbonate chemistry and cell densities

The performance of the continuous system can be measured by how close the actual culture pCO_2 was to the target pCO_2. Deviations from the target CO_2 concentration are mainly due to photosynthetic CO_2 draw-down, which is a function of the culture cell density and metabolic activity, and/or the accuracy of the CO_2-air mixture concentration.

Minimising the deviation of the experimental CO_2 concentration from the target concentration for each CO_2 treatment is vital for ocean acidification experiments. More critical for the detection of changes in biochemistry and physiology among CO_2 treatments, however, is the stability of CO_2 concentrations within each treatment and whether or not they overlapped. Standard deviations in pH and calculated pCO_2 for each treatment were relatively small using a continuous system (Table 4.1). The scatter in pCO_2 was larger in higher CO_2 treatments than in the control treatments (Figure 4.3) and this is likely due to small variations in air flow rates to which constant volumes of CO_2 gas were added. Variations in air flow rates will lead to larger variations in final pCO_2 in the high CO_2 treatments, where more CO_2 is added.

Table 4.1 Measured pH and calculated pCO_2 during the continuous culture experiments. Headings are target pCO_2 (µatm). SD = standard deviation, range = difference between highest and lowest recorded pH, measured pCO_2 values were calculated using CO2SYS from alkalinity and pH, positive (negative) values mean the actual CO_2 concentration was higher (lower) than the target value.

Fragilariopsis cylindrus	**390**	**570**	**750**	**950**
Average pH ± SD	8.02 ± 0.03	7.88 ± 0.03	7.77 ± 0.04	7.69 ± 0.03
pH range	0.13	0.15	0.20	0.17
Calculated pCO_2 ± SD	428 ± 34	590 ± 46	771 ± 67	950 ± 78
Difference from target	+ 38	+ 20	+ 21	± 0

Pyramimonas gelidicola	**390**	**570**	**750**	**950**
Average pH ± SD	8.04 ± 0.04	7.87 ± 0.03	7.75 ± 0.04	7.67 ± 0.04
pH range	0.17	0.13	0.16	0.17
Calculated pCO_2 ± SD	400 ± 41	612 ± 54	806 ± 80	977 ± 106
Difference from target	+ 10	+ 42	+ 56	+ 27

Phaeocystis antarctica	**390**	**570**	**750**	**950**
Average pH ± SD	8.02 ± 0.03	7.86 ± 0.04	7.76 ± 0.04	7.67 ± 0.03
pH range	0.14	0.15	0.19	0.14
Calculated pCO_2 ± SD	413 ± 31	644 ± 62	805 ± 76	993 ± 83
Difference from target	+ 23	+ 74	+ 55	+ 43

Cell densities reached approximately 265,000 cells/mL for *Fragilariopsis cylindrus* cultures, with an average growth rate of 0.47 d^{-1}, SE ± 0.02 and 14 generations through the course of the experiment. *Pyramimonas gelidicola* cultures reached approximately 45,300 cells/mL, had an average growth rate of 0.38 d^{-1}, SE ± 0.03 and went through 6 generations and *Phaeocystis antarctica* reached approximately 105,800 cells/mL with an average growth rate of 0.53 d^{-1}, SE ± 0.03 and went through 6 generations.

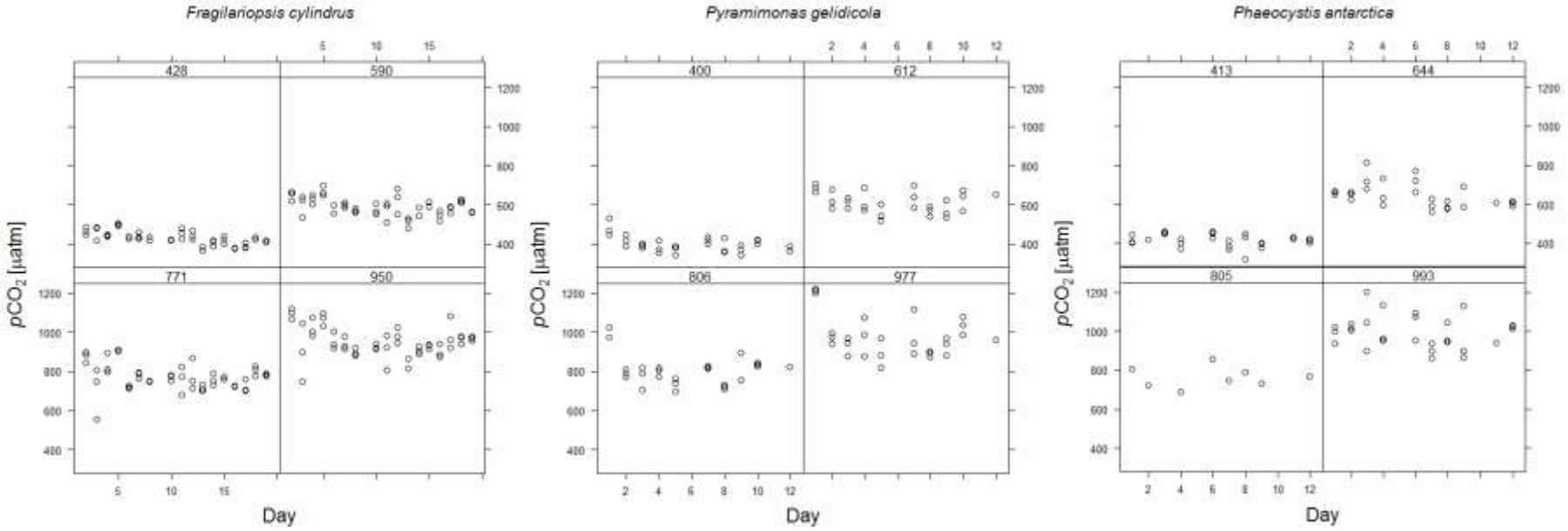

Figure 4.3 Calculated pCO_2 over time in the four CO_2 treatments of the three phytoplankton species. Average pCO_2 levels in µatm are indicated above each panel. Individual circles represent the three replicate culture vessels per treatment. (left) *Fragilariopsis cylindrus*; (centre) *Pyramimonas gelidicola*; (right) *Phaeocystis antarctica*.

4.4.2. Discussion

Our aim was to develop a phytoplankton culturing system that could facilitate fully replicated, long-term ocean acidification experiments with stable carbonate chemistry. Natural phytoplankton assemblages in the Southern Ocean experience large variations in seawater pH and pCO_2 (~300 µatm seasonally), in seasonal and diurnal cycles, particularly during intense blooms (McNeil *et al.* 2010; McNeil *et al.* 2011). Photosynthesis by prolific phytoplankton blooms in summer can reduce pCO_2 to approximately 100 µatm, while in autumn and winter the absence of light and upwelling of CO_2 rich deep water can increase pCO_2 to about 450 µatm (McNeil and Matear 2008; McNeil *et al.* 2011). Phytoplankton species exposed to such naturally variable CO_2 environments are likely to tolerate a broad range of pH and pCO_2. Indeed Berge *et al.* (2010) reported that diurnal changes in pH of 1 unit did not affect growth rates of a range of species in the laboratory, but sustained changes of such magnitude may elicit more significant responses as cells are not able to capitalise on intermittent favourable periods. Alternatively, cells may acclimate to the new pCO_2 environment and increase their tolerance. Arguably reasonably small changes in experimental pCO_2 due to natural processes like photosynthesis may be acceptable when trying to mimic natural surface ocean conditions (Shi *et al.* 2009). Yet, the aim of controlled laboratory experiments is to accurately maintain experimental conditions to detect biochemical and physiological changes among pCO_2 treatments.

Our continuous culture system was found to be a less labour intensive approach and provided stable carbonate chemistry conditions that compared well with other systems. The continuous culture experiment by Crawfurd *et al.* (2011), run over 12 weeks, was subject to a pH range of ~0.4 units. Crawfurd *et al.* (2011) controlled the carbonate chemistry by bubbling with CO_2-enriched air and by automated addition of pre-equilibrated media when the pH deviated by 0.01 units. Lefebvre *et al.* (2012) maintained cultures of the coccolithophore *Emiliania huxleyi* for close to 6 months in a cyclostat, where culture pH was maintained by continuously bubbling with CO_2-enriched air. Due to the labour intensity of their system they could not incorporate replication. Furthermore the actual pCO_2 levels of the cultures deviated greatly from their target values. The average pCO_2 of cultures with a target pCO_2 of 400 µatm averaged between 166 and 194 µatm and cultures aimed at 1000 µatm averaged between 308 and 367 µatm. The pH standard errors were between ±0.02 and ±0.04. Li and Campbell (2013) reported pH standard deviations of up to 0.07 using a turbidostat system. The continuous culture system we describe here maintained pCO_2 levels equally well, with little deviation from our target values and the ease of maintenance allowed full replication of up to four treatments (Table 4.1).

The Antarctic phytoplankton species used in our experiments at low temperatures had relatively low growth rates (0.4–0.5 d−1) compared to the species used by Crawfurd *et al.* (2011) (~0.9 d−1) and Lefebvre *et al.* (2012) (~0.9–1.3 d−1). The lower metabolic rates in phytoplankton in our study reduced biologically-induced variations to carbonate chemistry. Furthermore,

macronutrient concentrations mimicked the abiotic environment from which the phytoplankton were isolated. This kept cell densities at lower concentrations than would be supported by traditional, nutrient rich, culture media, and this also helped minimize changes in pCO_2 due to biological activity. To accommodate faster growing species, at potentially higher cell densities, either the pump rate of fresh CO_2-enriched media to the cultures and/or the volume of CO_2 gas added to the inflowing air would need to be increased. Both options can be easily facilitated in the above described system. A higher pump speed of inflowing media would also improve the accuracy with which media is delivered to each culture bag, as we were operating at the lowest limits of pump speed on our peristaltic pumps.

We did not test the robustness of our setup to the regular perturbations caused by day-night cycles in photosynthesis and respiration since we used continuous light throughout the experiments. Diurnal light cycles, though potentially more representative of the natural environment, induce diurnal cycles in the physiology and biochemistry of the algae (Hitchcock 1980). Laboratory studies have shown that diurnal variations in C:N:P were of similar magnitude to CO_2-induced differences (Burkhardt *et al.* 1999) and may mask the effects of experimental pCO_2 treatment. Furthermore, light dark cycles can cause large variations in the culture pCO_2 due to the changes in the ratio of photosynthesis to respiration. Lefebvre *et al.* (2012) measured significant daily variations between dark (580 µatm ±40 µatm) and light phase (340 µatm ±20 µatm) in cultures that were bubbled with CO_2-enriched air of 1000 ppm.

In order to simulate natural changes in carbonate chemistry, the culture medium can either be altered by addition of CO_2 as gas, as equimolar volumes of HCl and Na_2CO_3 (Riebesell *et al.* 2010) or in doses of pre-equilibrated seawater (McGraw *et al.* 2010). McGraw *et al.* (2010) developed a very stable (pH deviation of 0.02 units), individually adjustable and automated system for ocean acidification experiments with coralline algae. However, the system described by McGraw *et al.* (2010) requires a self-developed software and sophisticated electronics. Furthermore, since their culture vessels are flushed numerous times per hour to maintain stable pH in the presence of photosynthesis and calcification, this flow-through setup is not suitable for slow growing phytoplankton cultures. Therefore, we chose to bubble our cultures with CO_2-enriched air as recommended by Gatusso and Lavigne (2009), despite some reports of adverse effects of small-scale turbulence on the growth rates of delicate taxa (Peters *et al.* 2006; Schapira *et al.* 2006; Berdalet and Peters 2007). To avoid damage by bubbling of fragile species, the CO_2-enriched air can be continuously pumped into the headspace and will equilibrate from there into the underlying culture medium, especially when some form of agitation is provided. This works well for small culture vessels and low cell densities. For larger culture vessels, such as those used in this study, the surface to volume ratio is insufficient and rates of gas diffusion into the culture are too slow. Thus flushing the headspace with CO_2-enriched air is often insufficient to attain elevated pCO_2 in cultures. Bubbling CO_2-enriched air directly into the culture is an easy and simple alternative that also provides the culture with continuous agitation to reduce settling of cells on the bottom of the vessel (Wynn-Edwards *et al.* 2014a).

Fluctuations in CO_2 concentrations of the local ambient air can be a possible source of pCO_2 variation in experiments. While we did not find this to be an issue in our setup, CO_2 can be removed from the air source before adding the required volume of CO_2 to achieve the target concentration. For smaller volumes of air this can be done by passing the ambient air through a soda lime-packed column.

The peristaltic pumps in our system allowed two different species (housed in 12 separate cultures) to be studied simultaneously. If a precise estimate of growth rate is required, however, we recommend the use of individual pumps for each culture vessel. That way flush rates can be adjusted individually for each culture and influx of media and outflux of culture can be accurately monitored to calculate growth rates. However, this would greatly increase the cost of the system.

4.5. Conclusions

Phytoplankton form the basis of marine food webs and understanding the effects of enhanced CO_2 on their physiology, biochemical composition and abundance is vital to predict the effects of ocean acidification on marine ecosystems. Long-term laboratory based ocean acidification experiments are an important tool in answering these questions. A continuous culture system reduced the amount of work required to maintain the algae in exponential growth and, by continuous addition of media at the desired pH and pCO_2, stabilized the carbonate chemistry. Automatic continuous dilution and

addition of nutrients eliminated the requirement for manual dilutions, reduced disturbances to the culture by removal of large volumes of culture, and eliminated periodic changes in cell physiology with changing nutrient availability. The system described here provides several advantages over batch cultures, such as ease of maintenance, stable nutrient concentrations and carbonate chemistry at higher cell densities. However, its suitability depends on the experimental design and/or resources available. While automated dilution facilitates greater replication, the number of replicates is limited by the number of channels of the peristaltic pumps and the constant supply of sterile culture medium can become resource intensive. In summary, the continuous system we describe here is relatively inexpensive, and easy-to-use compared to other systems. It has proven effective for slow growing cultures at low temperatures and continuous light intensities, can easily be adjusted for faster growing species and represents an effective alternative to the use of batch experimental cultures.

5. Species – specific variations in the nutritional quality of Southern Ocean phytoplankton in response to elevated pCO_2

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5.1. Abstract

Increased seawater pCO_2 has the potential to alter phytoplankton biochemistry, which in turn may negatively affect the nutritional quality of phytoplankton as food for grazers. Our aim was to identify how Antarctic phytoplankton, *Pyramimonas gelidicola*, *Phaeocystis antarctica*, and *Gymnodinium sp.*, respond to increased pCO_2. Cultures were maintained in a continuous culture setup to ensure stable CO_2 concentrations. Cells were subjected to a range of pCO_2 from ambient to 993 µatm. We measured phytoplankton response in terms of cell size, cellular carbohydrate content, and elemental, pigment and fatty acid composition and content. We observed few changes in phytoplankton biochemistry with increasing CO_2 concentration which were species-specific and predominantly included differences in the fatty acid composition. The C:N ratio was unaffected by CO_2 concentration in the three species, while carbohydrate content decreased in *Pyramimonas gelidicola*, but increased in *Phaeocystis antarctica*. We found a significant reduction in the content of nutritionally important polyunsaturated fatty acids in *Pyramimonas gelidicola* cultures under high CO_2 treatment, while cellular levels of the polyunsaturated fatty acid 20:5ω3, EPA, in *Gymnodinium sp.* increased. These changes in fatty acid profile could affect the nutritional quality of phytoplankton as food for grazers, however, further research is needed to identify the mechanisms for the observed species-specific changes and to improve our ability to extrapolate laboratory-based experiments on individual species to natural communities.

5.2. Introduction

Human activities have led to an increase in atmospheric CO_2 concentration of which an estimated 30% have been absorbed by the oceans, causing global average surface seawater pH to drop by 0.1 units (Sabine *et al.* 2004; IPCC 2007a; Zeebe *et al.* 2008), a process termed ocean acidification (Caldeira and Wickett 2003; The Royal Society 2005). High latitudes will be particularly vulnerable due to their capacity to store more CO_2, and upwelling and subsequent entrainment of CO_2-rich deep waters during winter (IPCC 2007b; Fabry *et al.* 2008; McNeil and Matear 2008; Tortell *et al.* 2008; Fabry *et al.* 2009), rendering its inhabitants among the first to be affected by ocean acidification. Yet, little is known presently about the susceptibility of polar organisms to increased pCO_2, and this is particularly true for Antarctic phytoplankton (Riebesell 2004; Montes-Hugo *et al.* 2009).

Ocean acidification has the potential to alter phytoplankton biochemistry. Elevated CO_2 concentration has been shown to influence the ratio of carbon to nutrient uptake rates in phytoplankton (Burkhardt and Riebesell 1997; Riebesell *et al.* 2007; Bellerby *et al.* 2008; Paulino *et al.* 2008) and consequently an increase in C:N:P ratio (Iglesias-Rodriguez *et al.* 2008; Hoogstraten and Timmermans 2012; Schoo *et al.* 2013). A reduction in the percentage of polyunsaturated fatty acids (PUFA) in the diatom *Thalassiosira pseudonana* has also been reported (Rossoll *et al.* 2012).

Phytoplankton response to elevated CO_2 concentrations and lowered pH has been found to be species-specific and can lead to shifts in the species composition and bulk biochemical parameters of natural phytoplankton communities (Hinga 2002; Tortell *et al.* 2002; Kim *et al.* 2006; Tortell *et al.* 2008). Predicting how phytoplankton will respond to ocean acidification has therefore been a difficult task. Contrary to the hypothesis that climate change will fertilize the oceans via increased availability of CO_2 and thereby stimulate primary productivity, on a global scale ocean primary productivity has declined since the early 1980's (Gregg *et al.* 2003) and might continue to do so due to ocean warming (Boyce *et al.* 2010).

Alterations to the composition of phytoplankton communities, as well as individual species' nutritional quality and availability, could have major ramifications for higher trophic levels (Urabe *et al.* 2003; Carotenuto *et al.* 2007; Hauri *et al.* 2009; Urabe and Waki 2009). The fatty acid profile of phytoplankton is of particular interest as an indicator of their nutritional quality for grazers. Fatty acids are divided into PUFA, monounsaturated fatty acids (MUFA) and saturated fatty acids (SFA). The composition of PUFA and the ratio of PUFA to MUFA and SFA are critically important for grazer development, reproduction and hatch rates, as has been well researched by the aquaculture and other industries (Koven *et al.* 1989; Harrison *et al.* 1990; Carvalho and Malcata 2005; Bell *et al.* 2007; Yoshida *et al.* 2011; Chen *et al.* 2012).

To understand why and how phytoplankton communities change under the influence of ocean acidification, we need to understand the nature and implications of individual species' changes and what the common and species-specific changes are. Therefore, the aim of this research was to establish whether the biochemistry of Antarctic phytoplankton responds to elevated CO_2 concentrations and if so, to identify similarities or differences in this response amongst species. Based on literature reports our hypothesis was to find an increase in C:N ratio, a decrease in PUFA and a decrease in cellular carbohydrate contents (Thornton 2009) with increasing pCO_2.

5.3. Materials and Methods

We conducted two experiments in which three Antarctic phytoplankton species were exposed to CO_2 concentrations ranging from ambient to 993 µatm following recommendations by Barry *et al.* (2010). The species chosen were the prasinophyte, *Pyramimonas gelidicola*, a single cell strain of the haptophyte *Phaeocystis antarctica*, and the dinoflagellate *Gymnodinium sp.* We chose these species to represent a mixture of phytoplankton types, commonly found in the waters off Davis Station, Antarctica, where these species were isolated from. We measured their pigment and fatty acid profiles, particulate carbohydrate content and carbon to nitrogen ratio immediately after acclimation to the experimental conditions. In agreement with recommendations in best practice guides (Riebesell *et al.* 2010), we used a continuous culture system with CO_2-enriched gas aeration to achieve stable carbonate chemistry that mimics the natural changes occurring due to ocean

acidification. For a better understanding of how individual species will respond under natural conditions, we conducted our experiments at macronutrient concentrations reported for the area from which our strains were isolated (near shore coastal waters off Davis Station, Antarctica) and set the light intensity to a similarly high level, as light intensity can greatly alter the CO_2-induced response of phytoplankton (Chen and Gao 2011; Gao *et al.* 2012; Li and Campbell 2013). Furthermore, we chose to expose all species to continuous light to minimize diurnal variations which can mask CO_2-induced changes (Burkhardt *et al.* 1999).

We used a continuous culture system with CO_2-enriched gas aeration to achieve a stable carbonate chemistry that mimics the natural changes occurring due to ocean acidification, following the recommendations by Riebesell *et al.* (2010). The main advantages of a continuous culture system with automatic constant dilution are: (1) the reduced labour intensity compared to semi-continuous culturing, that require regular manual dilutions; and (2) the possibility to use low nutrient concentrations that are approximating natural conditions compared to artificially high nutrient concentrations, such as in the commonly used full-strength f/2 medium. We chose a base recipe of f/2 medium (Guillard and Ryther 1962; Guillard 1975) as it lacks any buffer that could affect the carbonate chemistry and trace metal speciation in the growth medium, which would thereby affect medium pH and phytoplankton growth (Shi *et al.* 2009). Iron concentrations were not adjusted to the realistically low concentrations of the Southern Ocean since iron limitation has been found to influence phytoplankton response to elevated CO_2 concentrations (Sugie and

Yoshimura 2013); such changes would add a further level of complexity in interpreting our results. Nitrate and phosphate were adjusted to concentrations reported around O'Gorman Rocks, off Davis Station, Antarctica (Gibson 1998; Roden *et al.* 2013). Silicate concentrations were lower than around O'Gorman Rocks but not limiting (Harrison *et al.* 1977; Egge and Aksnes 1992). To achieve the target pCO_2, we bubbled the phytoplankton bag cultures with air containing predetermined CO_2 concentrations. Bubbling is a simple and effective way of altering the carbonate chemistry in agreement with natural changes (Gattuso and Lavigne 2009). The culture system is described in detail elsewhere (Wynn-Edwards *et al.* 2014b) and will be summarized briefly below.

The system housed 24 × 2.3 L custom-made, transparent plastic bags (polyethylene, Entapack, Australia) thereby allowing two phytoplankton species to be studied simultaneously; each species was examined with triplicate bags exposed to four CO_2 treatments (three CO_2 enriched treatments and an ambient control). For each of the four target CO_2 concentrations, two 28 L media reservoirs were bubbled with 0.2 μm filtered air. The reservoirs were used to supply fresh, sterile (0.2 μm filtered) medium by two 12-channel peristaltic pumps (Masterflex, John Morris Scientific Pty Ltd, Chatswood, NSW, Australia) to the culture bags corresponding to that pCO_2 treatment in order to maintain the cells in exponential growth (Fig 5.1). Each culture bag was inoculated with a clonal phytoplankton culture of the same parent population and randomly attributed to the treatments. Initial cell density for *Pyramimonas gelidicola* cultures was ~8000 cells/mL, ~9500 cells/mL for *Phaeocystis*

antarctica cultures and only ~80 cells/mL for *Gymnodinium sp.* cultures. The peristaltic pumps flushed the culture bags with sterile nutrient medium at a rate equivalent to the species' growth rate once each species had reached exponential growth and sufficient cell density (Fig 5.2). *Gymnodinium sp.* was not constantly diluted before reaching the goal of 6–7 generations of acclimation at the end of the experiment, due to its slow growth rate.

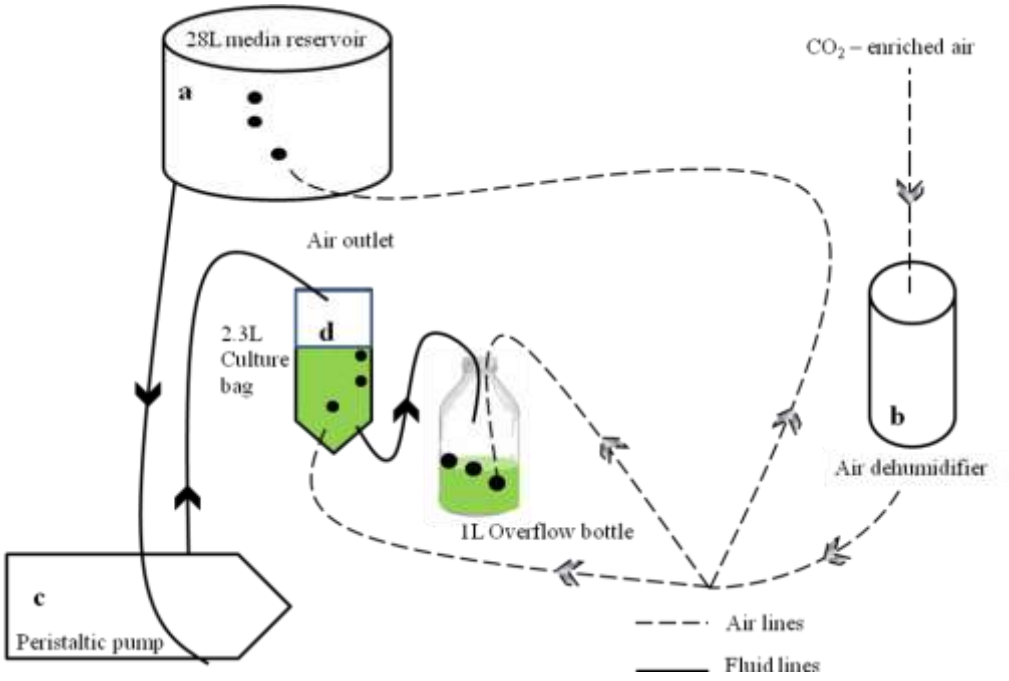
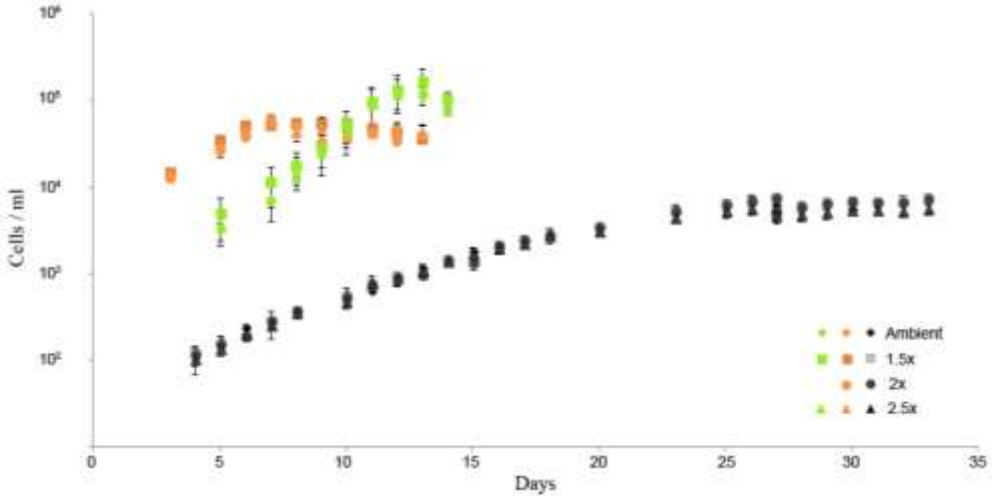


Figure 5.1 Overview schematic diagram of the ocean acidification continuous culture system. For simplicity only one CO_2 treatment and culture bag is shown. (a) Eight 28 L media reservoirs supplied the cultures with media of the respective pCO_2. The reservoirs were continuously bubbled with CO_2-enriched air of the respective concentration; (b) Air bubbling through the reservoirs and all culture bags and overflow bottles was first dehumidified by passing through a silica-gel filled cylinder fitted with an activated charcoal stage at the end to remove any organic contaminants; (c) Peristaltic pumps delivered the media from the reservoirs to each culture bag at a rate equivalent to the growth rate of the culture; (d) Culture bags and overflow bottles were continuously bubbled to maintain stable carbonate chemistry. At the same rate as media was pumped in, culture overflowed into a sterile overflow bottle, which was sampled for subsequent biochemical and other analyses.

Figure 5.2 Daily cell densities with standard errors of *Pyramimonas gelidicola* (orange), *Phaeocystis antarctica* (green) and *Gymnodinium sp.* (black) cultures under the different CO_2 treatments.



The out-flowing culture was collected in sterile 1 L glass overflow bottles, providing culture material for subsequent analyses (Fig 5.1), as has been performed elsewhere (Schoo *et al.* 2013). Use of an overflow vessel to collect surplus culture allowed us to measure a suite of biochemical parameters for which large volumes of culture were required (up to 700 mL). By using the out-flowing culture rather than sample from within the culture bag, we avoided the possibility of contamination and also avoided any disturbances to the culture physiology by removing more than 10% of the culture volume (Feng *et al.* 2009). Due to the slow growth rate of our three phytoplankton species and thus the slow dilution rate, surplus culture was left to accumulate for up to 2 days to provide the large volumes needed for the biochemical assays. These overflow bottles were located adjacent to the culture to ensure that the overflow bottles received the same light levels and temperatures as the cultures. Furthermore, the overflow bottles were bubbled with the same CO_2-air mix until sampling occurred. Thus the only property in the overflow

bottles that potentially differed from conditions within the continuous system was the nutrient concentration. We cannot rule out the possibility that nutrient concentrations in the overflow bottles decreased over the 2 day period. However, it has been shown that diatoms have the ability to store nitrogen within the cells (Lomas and Gilbert 2000 and references therein) and given the slow doubling time (~2 days) of our selected phytoplankton species, only those cells entering the overflow bottle at the start of the 2 day period would have undergone a maximum of one generation outside of the continuous culture system *versus* at least five generations of acclimation within the continuous culture system. Further, cell densities within the overflow bottles never exceeded the carrying capacity associated with our culture medium, as established prior to the experiments. Based on this, we have assumed that samples taken from the overflow bottles are representative of the culture within the continuous culture system. However, this uncertainty could be addressed in future experiments by monitoring nutrient concentrations in the overflow bottle as well as increasing culture bag volumes to shorten the timeframe required to accumulate enough culture volume for biochemical analyses.

5.3.1. Experimental conditions

The cultures, overflow bottles and media reservoirs were kept in a temperature controlled refrigerator, maintained at an average 2.9 °C. The culture bags were positioned in front of fluorescent lights with an irradiance of 267 ± 6.9 µmol m^{-2} s^{-1}, approximating the light intensities at 5 m water depth around Davis Station, East Antarctica, from where most of these species were isolated

(Thomson *et al.* 2008). Each culture, medium reservoir and overflow bottle was continuously bubbled with CO_2-regulated air, using mass flow controllers (Horiba STEC SEC-E-40), which mixed pure food grade CO_2 (BOC, Hobart, TAS, Australia) with ambient air to achieve specific CO_2 concentrations for the treatments. The resulting gas was passed through silica gel, activated charcoal, and a 0.2 μm filter to remove any contaminants before being used to adjust the CO_2 content of the culture bags, reservoirs and overflow bottles. Experimental conditions are listed in Table 5.1.

Table 5.1 Experimental conditions for all phytoplankton species. Values are averages with standard deviations, SD, in brackets. NO_x includes NO_3^- and NO_2^-.

		Nutrient concentrations [µM]			**CO_2 concentration [µatm]**			
Species	**Acclimation in generations**	**NO_x**	**Si**	**P**	**Ambient**	**1.5x**	**2x**	**2.5x**
Pyramimonas gelidicola	~ 5	17.5 (± 4.5)	24.4 (± 5.2)	5.7 (± 1.3)	400 (±41)	612 (±54)	806 (±80)	977 (±106)
pH ± SD					8.04 (±0.04)	7.87 (±0.03)	7.75 (±0.04)	7.67 (±0.04)
Phaeocystis antarctica	~ 6	17.5 (± 4.5)	24.4 (± 5.2)	5.7 (± 1.3)	413 (±31)	644 (±62)	-	993 (±83)
pH ± SD					8.02 (±0.03)	7.86 (±0.04)	-	7.67 (±0.03)
Gymnodinium sp.	~ 6	20.9 (± 1.8)	17.6 (± 0.5)	1.5 (± 0.2)	458 (±48)	580 (±49)	797 (±79)	973 (±82)
pH ± SD					8.02 (± 0.04)	7.89 (± 0.03)	7.75 (± 0.04)	7.70 (± 0.03)

5.3.2. Carbonate chemistry

Carbonate chemistry in the culture bags was monitored daily by measuring pH with a Mettler Toledo Multi Seven pH meter (Mettler Toledo, Australia). The pH meter was calibrated daily using freshly prepared tris- and aminopyridine buffers made in artificial seawater according to the SOP 6a in "Guide to best practices for ocean CO_2 measurements" (Dickson *et al.* 2007). Alkalinity samples (50 mL) were taken at regular intervals, poisoned with 25 µL saturated mercuric chloride solution and stored refrigerated in the dark until analysis in a closed cell using a Total Alkalinity Titrator ATT-05 (Kimoto, Osaka, Japan). A temperature probe logged the air temperature inside the refrigerator every 30 min (data not shown). CO_2 concentrations were calculated with the CO2SYS.BAS Excel programme (Lewis and Wallace 1998) based on total alkalinity, pH, temperature, salinity and nutrient concentrations using the constants after Mehrbach *et al.* (1973) as refitted after Dickson and Millero (1987).

5.3.3. Physiological and biochemical analyses

After acclimation of the cultures of each phytoplankton species to the four CO_2 treatments, cultures in the overflow bottles were filtered to provide samples for fatty acid, pigment and C:N composition, and particulate carbohydrate content. Problems with the *Phaeocystis antarctica* cultures of the 2× ambient CO_2 treatment lead to their exclusion from the data set and the 1.5× and 2.5× CO_2 treatments were only comprised of two replicates each.

Cell abundance

Daily cell abundance samples were taken to monitor exponential growth and relate measurements to a per cell basis. Samples were analysed using a BD FACSCalibur cytometer (Becton Dickson, San Diego, CA, USA) equipped with a 488 nm argon laser and cell concentrations were calculated by dividing event counts from bivariate scatter plots by the volume of culture analysed. PeakFlow Green 2.5 µm beads (Molecular Probes, Invitrogen, Mulgrave VIC, Australia) were added to each sample to monitor fluorescent signal strength.

Cell dimensions

Cell dimensions were measured on Field Emission Scanning Electron Microscope images (JEOL JSM6701F, Frenchs Forest, NSW, Australia). For cells of *Gymnodinium sp.* and *Pyramimonas gelidicola* length and width were measured. Cell dimensions of *Phaeocystis antarctica* cells were not measured.

Pigments

Samples of each culture were vacuum filtered onto 13 mm GF/F filters (Whatman, GE Healthcare Life Sciences, Rydalmere, NSW, Australia), blotted dry and immediately frozen in liquid N_2 in cryovials. The samples were stored at −135 °C until analysis using a modified method (Mock and Hoch 2005). Pigments were extracted with 300 µL dimethylformamide with 50 µL methanol containing 140 ng of apo-8'-carotenal (Fluka) internal standard and analysed by high pressure liquid chromatography (HPLC) (Zapata *et al.* 2000) using a Waters 626 pump, Gilson 233 XL autoinjector,

Waters Symmetry C8 column, a Waters 996 diode array detector and a Hitachi FT1000 fluorescence detector. Pigments were identified by comparison of their retention times and spectra with a sample of mixed standards from known cultures (Wright and Jeffrey 1997) injected at the start of each daily sample queue. Peaks were integrated using Waters Empower software, manually checked and corrected where necessary and quantified using an internal standard method (Mantoura and Repeta 1997). Further details on the procedures are in (Wright *et al.* 2010).

Cellular carbohydrates and carbon to nitrogen ratio

Each culture was filtered onto a muffled quartz filter (Sartorius, Goettingen, Germany) and ¼ was used for C:N ratio analysis and the remaining ¾ for carbohydrate analysis. Cell distribution across the filters was even, but to minimize bias between the analyses through possible uneven filtration, the filters were cut symmetrically into eight pieces and opposite ⅛ pieces of the filter were used to amount to the ¼ used for elemental analysis.

Phytoplankton particulate organic carbohydrates were first denatured into monosaccharides (adapted from Brown *et al.* 1998) and the carbohydrate content determined via a standard colorimetric analysis (Dubois *et al.* 1956) on a GBC UV-Vis 916 spectrophotometer (GBC Scientific Equipment, Braeside, VIC, Australia).

Prior to C:N analysis, inorganic carbon was removed with 2 molar HCl. C:N composition was determined via a Thermo Finnigan EA 1112 Elemental Analyser (CEInstruments Ltd, Lancashire, UK).

Fatty acid analysis

Culture was filtered on pre-extracted (1:1 v/v chloroform:methanol) 25 mm GF/F (Whatman, GE Healthcare Life Sciences, Rydalmere, NSW, Australia) filters. Filters were trans-methylated to produce fatty acid methyl esters (FAME) by heating in methanol:chloroform:concentrated hydrochloric acid (3 mL, 10:1:1 v/v/v) at 80 °C for 2 h (Christie 1982). FAME were extracted into hexane:chloroform (4:1 v/v) and concentrated under a stream of nitrogen. An internal injection standard (19:0 FAME) was added and the fatty acid composition analysed by gas chromatography using an Agilent Technologies 7890A gas chromatography (Palo Alto, CA, USA) fitted with an Equity −1 fused silica capillary column (15 m × 0.1 mm internal diameter, 0.1 μm film thickness), a flame ionization detector, a split/splitless injector and an Agilent Technologies 7683B Series auto sampler and injector. Helium was used as carrier gas. Operating conditions were as described in (Yoshida *et al.* 2011).

Peaks were quantified with Agilent Technologies ChemStation software (Palo Alto, CA, USA) and individual components were identified using mass spectral data and comparison of retention time data with those obtained for authentic and laboratory standards. Gas chromatography-mass spectrometric analyses were performed on a Finnigan Thermoquest GCQ gas chromatography-mass spectrometer fitted with an on-column injector using

Thermoquest Xcalibur software (Austin, TX, USA) and a capillary column of similar polarity to that described above.

5.3.4. Statistical analysis

Biochemical results were analysed with a linear mixed effects model, including the interaction of culture bag and culture position in the three rows as nested random effects. Significant differences between treatment cultures and control cultures in all tests were accepted at $p \leq 0.05$. Calculations were performed with the R software environment 2.14.2 (R Development Core Team 2012).

5.4. Results and Discussion

Cell dimensions and growth rates

Pyramimonas gelidicola cells, grown at elevated CO_2 concentrations, were not significantly different from their respective control cultures. *Gymnodinium sp.* cell dimensions were not affected by CO_2 concentration (Table 5.2).

Growth rates were not affected by CO_2 concentrations in any of the three species (Fig 5.2). Based on the exponential growth phase prior to culture dilutions, growth rates of our cultures were:

Pyramimonas gelidicola 0.38 d^{-1}, SE ± 0.03

Phaeocystis antarctica 0.53 d^{-1}, SE ± 0.03

Gymnodinium sp. 0.24 d^{-1}, SE ± 0.01

Table 5.2 *Pyramimonas gelidicola* and *Gymnodinium sp.* cell dimensions of the respective CO_2 treatments. SD in brackets, n = number of samples.

CO_2 treatment	**Ambient**	**1.5x**	**2x**	**2.5x**
	Pyramimonas gelidicola			
Length [µm]	6.72 (±1.01)	7.04 (±1.23)	6.82 (±1.03)	7.01 (±1.37)
Width [µm]	5.58 (±0.59)	5.80 (±0.64)	5.73 (±0.55)	5.70 (±0.72)
	n = 60	n = 60	n = 60	n = 60
	Gymnodinium sp.			
Length [µm]	16.62(±2.60)	17.21 (±2.65)	17.24 (±2.44)	16.54 (±2.42)
Width [µm]	8.16 (±1.08)	8.59 (±1.49)	8.97 (±1.63)	8.07 (±1.37)
	n = 45	n = 45	n = 58	n = 57

Pigments

There were no significant changes in pigment contents and ratios with increasing CO_2 levels in the three phytoplankton species (Table 5.3).

Table 5.3 Phytoplankton pigment ratios and contents in pg $cell^{-1}$ of the respective CO_2 treatments. Chl = Chlorophyll, ant = antheraxanthin, violax = violaxanthin, zea = zeaxanthin, Ddx = diadinoxanthin, dtx = diatoxanthin, SD in brackets, n = number of samples.

CO_2 treatment	**Ambient**	**1.5x**	**2x**	**2.5x**
		Pyramimonas gelidicola (n=3)		
Chl a	0.62 (±0.18)	0.47 (±0.11)	0.53 (±0.14)	0.70 (±0.06)
Chl a + b	0.75 (±0.22)	0.58 (±0.14)	0.66 (±0.18)	0.86 (±0.07)
(ant+violax+zea) / Chl a	0.32 (±0.02)	0.32 (±0.03)	0.36 (±0.01)	0.32 (±0.01)
γ-carotene / Chl a	0.04 (±0.004)	0.04 (±0.001)	0.04 (±0.002)	0.04 (±0.001)
Lutein / Chl a	0.12 (±0.01)	0.14 (±0.03)	0.16 (±0.01)	0.12 (±0.001)
		Phaeocystis antarctica (ambient n=3, 1.5x CO_2 n=2, 2.5x CO_2 n=2)		
Chl a	0.15 (±0.06)	0.10 (±0.02)	-	0.08 (±0.02)
Chl a + c2 + c3	0.17 (±0.08)	0.12 (±0.03)	-	0.09 (±0.03)
(Ddx + dtx) / Chl a	0.49 (±0.18)	0.68 (±0.18)	-	0.86 (±0.01)
		Gymnodinium sp. (n=3)		
Chl a	1.71 (±0.84)	2.32 (±0.06)	2.07 (±0.22)	1.95 (±0.71)
Chl a + c2	1.80 (±0.89)	2.43 (±0.06)	2.19 (±0.23)	2.04 (±0.74)
(Ddx + dtx) / Chl a	0.82 (±0.12)	0.77 (±0.07)	0.77 (±0.11)	0.84 (±0.09)
β-carotene / Chl a	0.07 (±0.01)	0.06 (±0.02)	0.04 (±0.02)	0.04 (±0.04)

Elemental composition

There was no significant change in elemental composition of the three phytoplankton species cells under the different CO_2 treatments (Table 5.4), although all cultures had lower %C and C:N ratios compared to control cultures at the highest CO_2 concentration.

Table 5.4 Phytoplankton elemental composition of the respective CO_2 treatments. Percentages of dry mass, SD in brackets, n = number of samples.

CO_2 treatment	**Ambient**	**1.5x**	**2x**	**2.5x**
		Pyramimonas gelidicola		
%C	10.7 (±2.2)	6.0 (±2.6)	8.1 (±9.8)	7.7 (±3.3)
%N	1.1 (±0.1)	0.7 (±0.0)	0.9 (±0.6)	0.9 (±0.1)
C:N	9.9 (±2.4)	8.6 (±4.1)	6.6 (±5.6)	8.5 (±3.9)
	n = 3	n = 3	n = 3	n = 3
		Phaeocystis antarctica		
%C	11.8 (±5.4)	13.0 (±5.4)	-	9.4 (±5.3)
%N	1.2 (±0.03)	1.1 (±0.1)	-	1.4 (±0.6)
C:N	9.4 (±4.1)	11.6 (±4.1)	-	6.7 (±1.0)
	n = 3	n = 2	-	n = 2
		Gymnodinium sp.		
%C	14.1 (±2.6)	14.2 (±2.8)	15.6 (±4.5)	10.5 (±2.5)
%N	2.0 (±0.2)	2.2 (±0.6)	2.0 (±0.3)	1.8 (±0.4)
C:N	6.9 (±0.9)	6.8 (±1.5)	7.6 (±1.1)	5.7 (±0.3)
	n = 3	n = 3	n = 3	n = 3

Particulate carbohydrates

Particulate organic carbohydrate (CHO) content per cell in *Pyramimonas gelidicola* cultures were significantly lower at 2× and 2.5× pCO_2 than in control cells ($p < 0.05$) (Fig 5.3 and Table 5.5). In *Phaeocystis antarctica* cultures, CHO content were significantly higher in the highest CO_2 treatments than in control cultures ($p < 0.05$). CHO content per cell in *Gymnodinium sp.* cultures were not affected by elevated CO_2 concentrations.

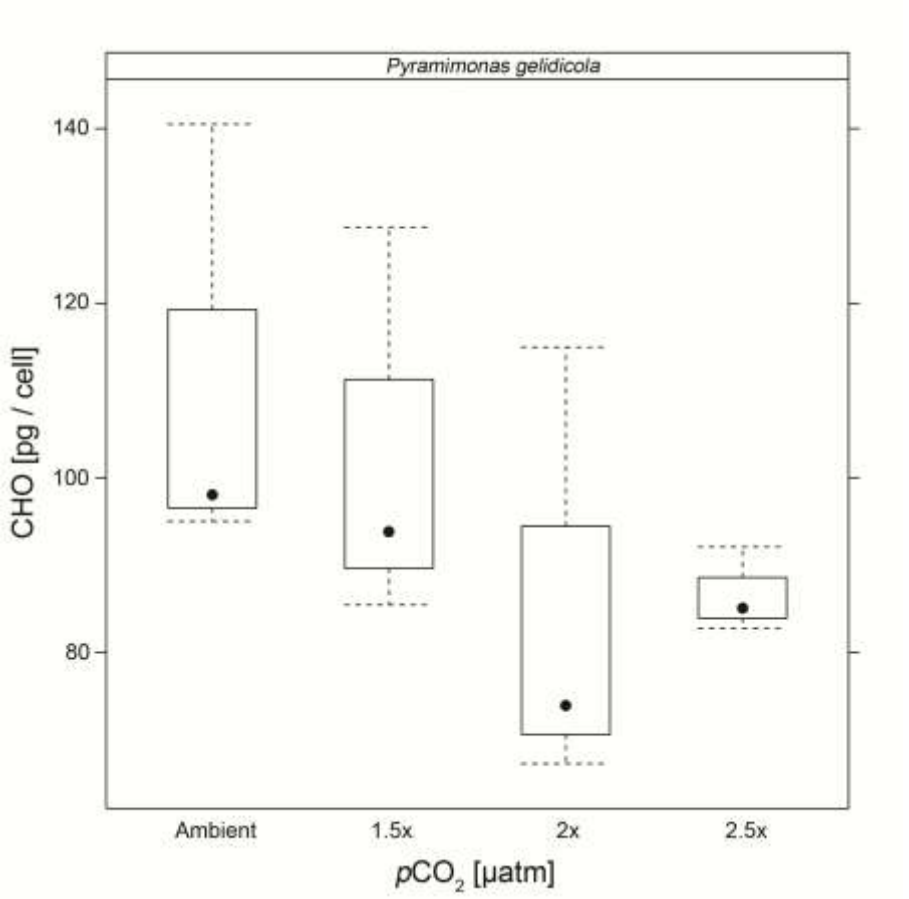


Figure 5.3 Cellular carbohydrate (CHO) content (pg/cell) with standard deviation of *Pyramimonas gelidicola* cultures under the different CO_2 concentrations.

Table 5.5 Phytoplankton particulate organic carbohydrate (CHO) contents in pg $cell^{-1}$ of the respective CO_2 treatments. SD in brackets, n = number of samples. *p<0.05

CO_2 treatment	Ambient	1.5x	2x	2.5x
	Pyramimonas gelidicola			
CHO	111.2 (±25.5)	102.7 (±22.9)	* 85.4 (±25.8)	* 86.7 (±4.9)
	n = 3	n = 3	n = 3	n = 3
	Phaeocystis antarctica			
CHO	7.6 (±0.5)	8.3 (±0.7)	-	* 9.9 (±0.5)
	n = 3	n = 2	-	n = 2
	Gymnodinium sp.			
CHO	93.4 (±30.3)	92.1 (±12.3)	110.4 (±26.5)	91.9 (±16.5)
	n = 3	n = 3	n = 3	n = 3

Fatty acids

Phaeocystis antarctica cultures showed no changes in the total amount of fatty acids per cell, the sum of monounsaturated fatty acids (MUFA), saturated fatty acids (SFA) and polyunsaturated fatty acids (PUFA), nor the sum of ω3 and ω6 PUFA, particularly DHA and EPA (Table 5.6). *Pyramimonas gelidicola* cultures showed decreased PUFA, ω3 and ω6 PUFA, DHA and total fatty acid content per cell at 2.5× pCO_2 (Table 5.6), but did not differ from control cultures in SFA and EPA content.

The cellular PUFA, MUFA, SFA, ω3 and ω6 PUFA and total fatty acid content of *Gymnodinium sp.* cultures were unaffected by CO_2. EPA contents per cell

were significantly elevated at all high CO_2 treatments compared to control cultures (Fig 5.4 and Table 5.6).

Table 5.6 Fatty acid content in pg/cell for all three phytoplankton species at the respective CO_2 treatments. SD in brackets, n = number of samples. *p<0.05, **p<0.01

CO_2 treatment	**Ambient**	**1.5x**	**2x**	**2.5x**
		Pyramimonas gelidicola		
SFA	0.3 (±0.08)	0.4 (±0.12)	0.4 (±0.12)	0.3 (±0.21)
MUFA	3.0 (±0.36)	4.6 (±0.92)*	3.8 (±0.72)	2.2 (±0.85)
PUFA	9.9 (±1.10)	9.4 (±0.90)	9.5 (±1.38)	6.5 (±2.72)*
ω3	4.9 (±0.67)	4.3 (±0.36)	4.7 (±0.63)	3.4 (±1.29)*
ω6	2.5 (±0.31)	2.7 (±0.34)	2.6 (±0.47)	1.5 (±1.00)*
DHA	1.2 (±0.19)	1.1 (±0.10)	1.2 (±0.15)	0.8 (±0.36)*
EPA	0.1 (±0.01)	0.1 (±0.02)	0.1 (±0.01)	0.1 (±0.03)
Total FA	14.9 (±1.5)	16.3 (±2.3)	15.4 (±2.2)	10.2 (±3.9)*
	n = 3	n = 3	n = 3	n = 3
		Phaeocystis antarctica		
SFA	0.2 (±0.15)	0.2 (±0.03)	-	0.3 (±0.06)
MUFA	0.7 (±0.58)	0.5 (±0.17)	-	0.7 (±0.11)
PUFA	1.9 (±1.42)	1.4 (±0.04)	-	1.6 (±0.25)
ω3	1.1 (±0.81)	0.8 (±0.00)	-	0.9 (±0.13)
ω6	0.8 (±0.61)	0.6 (±0.04)	-	0.7 (±0.11)
DHA	0.5 (±0.37)	0.4 (±0.02)	-	0.5 (±0.08)
EPA	0.1 (±0.09)	0.1 (±0.00)	-	0.1 (±0.01)
Total FA	3.1 (±2.37)	2.3 (±0.28)	-	2.9 (±0.48)
	n = 3	n = 2	-	n = 2
		Gymnodinium sp. (n=3)		
SFA	28.0 (±16.47)	26.4 (±3.93)	35.4 (±30.07)	20.0 (±9.40)
MUFA	27.0 (±8.65)	27.0 (±2.86)	* 37.0 (±7.98)	31.2 (±5.31)
PUFA	80.6 (±38.52)	96.8 (±7.58)	117.1 (±52.75)	94.1 (±27.36)
ω3	33.7 (±12.41)	39.7 (±2.55)	44.7 (±14.12)	38.2 (±10.79)
ω6	47.0 (±26.42)	57.2 (±5.14)	72.6 (±38.80)	55.7 (±16.60)
DHA	18.0 (±7.75)	22.0 (±1.92)	25.3 (±9.77)	22.6 (±5.13)
EPA	0.17 (±0.061)	0.26(±0.036)**	0.24(±0.020)**	0.31(±0.058)**
Total FA	153.6 (±72.45)	170.5 (±11.91)	216.2(±104.09)	164.9 (±44.92)
	n = 3	n = 3	n = 3	n = 3

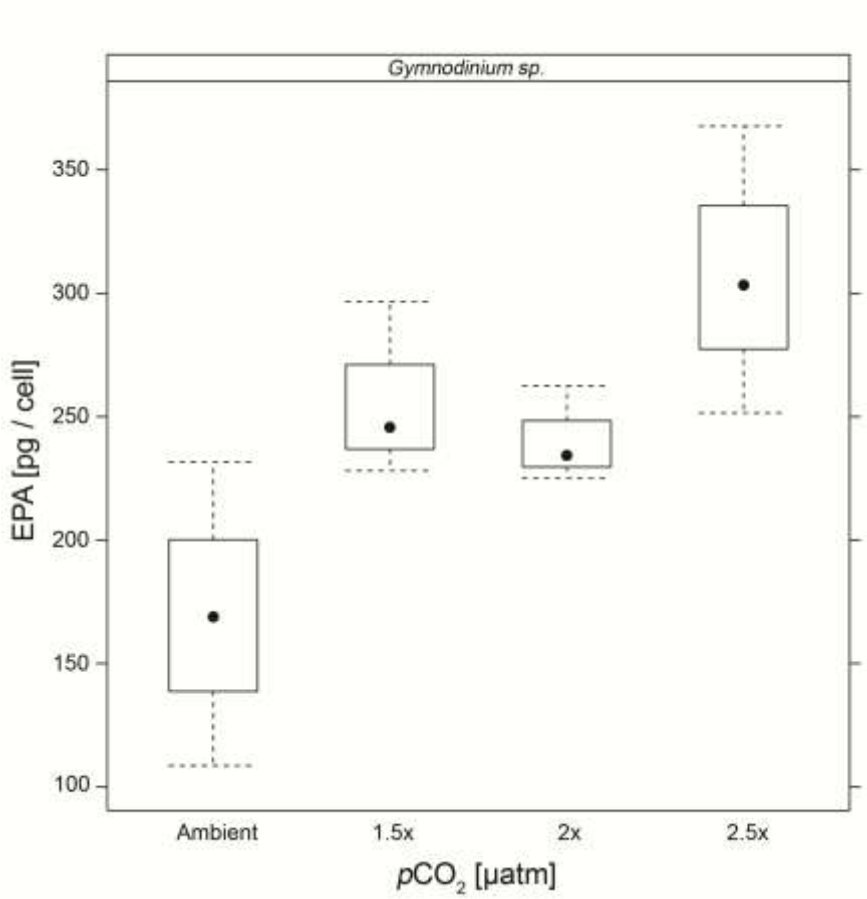


Figure 5.4 EPA content (pg/cell) with standard deviation of *Gymnodinium sp.* cultures under the different CO_2 concentrations.

Based on literature reports, our hypothesis was that an increase in C:N ratio, a decrease in PUFA and a decrease in cellular carbohydrate contents may occur with increasing pCO_2. Between the three Antarctic phytoplankton species examined here, we found subtle but highly variable responses under elevated CO_2 concentrations, with opposite changes observed in cellular carbohydrate contents as well as in fatty acid profiles.

Contrary to our hypothesis we found no changes in C:N ratio in the three phytoplankton species examined. This is consistent with some literature results (Burkhardt and Riebesell 1997; Burkhardt *et al.* 1999), which showed only minor responses of C:N ratios in a range of species under CO_2

concentrations above present day levels, but it is in contrast to other studies that have shown increased C:N ratios under elevated pCO_2 (Iglesias-Rodriguez *et al.* 2008; Hoogstraten and Timmermans 2012; Schoo *et al.* 2013). A species-specific and even life stage-specific response of C:N ratio to elevated pCO_2 has been reported elsewhere (Fiorini *et al.* 2010).

Cellular carbohydrate content of the prasinophyte, *Pyramimonas gelidicola*, and the haptophyte, *Phaeocystis antarctica*, cultures showed significant changes under elevated CO_2 concentrations. In agreement with the findings of Thornton [36], who studied the marine diatom *Chaetoceros muelleri*, cellular carbohydrate contents of *Pyramimonas gelidicola*, cultures decreased about 22% under 2× (pH = 7.75) and 2.5× (pH = 7.67) ambient CO_2 concentrations, compared to a ~20% decrease at pH 7.9 reported by Thornton (2009). Extracellular carbohydrate content was not measured in our experiment, thus we cannot exclude the possibility of excess carbon being accumulated and excreted as transparent exopolymers or low molecular weight carbohydrates in *Pyramimonas gelidicola* cultures, as suggested by Engel (2002) and Thornton (2009). Cellular carbohydrate content increased by ~30% in *Phaeocystis antarctica* cultures at 2.5× ambient pCO_2 (pH = 7.67). This is a larger increase than the 23% increase in cellular glucan content, a storage carbohydrate, reported in the marine diatom *Skeletonema costatum* grown at pH 7.5 (Taraldsvik and Myklestad 2000). Taraldsvik and Myklestad (2000) manipulated the pH of the growth medium via addition of acid/base in contrast to the use of CO_2 as occurred here, which might explain the difference in the magnitude between our result and theirs. This opposing trend

in cellular carbohydrate contents between our two species highlights the importance of analysing the changes in both particulate and dissolved carbohydrates in future studies to identify the fate of C in the cellular and extracellular C pool. It also highlights the possibility that different phytoplankton types respond differently to increased CO_2 concentrations. However, as the results by Thornton (2009) and Taraldsvik and Myklestad (2000) show, even within the same phytoplankton types, in this case diatoms, opposing response to CO_2 has been found.

Equally, no universal response in fatty acid content to increased pCO_2 was found in the three phytoplankton species investigated here. However, the changes in fatty acid profiles found in *Pyramimonas gelidicola* are of particular interest with regards to the availability of essential fatty acids for grazers. A decrease in PUFA content could constitute deterioration in the nutritional quality of phytoplankton for grazers, while an increase in these essential fatty acids such as EPA in *Gymnodinium sp.* could improve their quality as food. The effects of ocean acidification on the nutritional quality of phytoplankton are still mostly unknown with only a few studies conducted to date. While Leu *et al.* (2013) could not find any deterioration in a natural plankton community in the Arctic, Rossoll *et al.* (2012) reported that *Thalassiosira pseudonana* grown at high pCO_2 negatively affected growth and reproduction of the copepod *Acartia tonsa*. Similarly Wynn-Edwards *et al.* (submitted) found negative effects of *Pseudonitzschia subcurvata* grown at high pCO_2 on Antarctic krill, *Euphausia superba*, larval mortality rates. The PUFA content in *Pyramimonas gelidicola* cultures under elevated CO_2 was

about 34% lower than under ambient conditions and this agrees well with the 36% decrease in PUFA content in the diatom *Thalassiosira pseudonana* under 761 µatm pCO_2 reported by Rossoll *et al.* (2012). Detailed studies of the mechanisms and pathways of lipid and fatty acid production in other organisms suggest that external and internal pH influence lipid and fatty acid production. A decrease in external pH can translate into a decrease in internal pH (Lane and Burris 1981). Decreased internal pH in turn was reported to suppress phospholipid metabolic genes in yeasts (Young *et al.* 2010) and a lower degree of unsaturation of fatty acids in CO_2-enriched cultures of *Chlorella kessleri* compared to ambient CO_2 concentrations was at least partially attributed to suppressed fatty acid synthesis and thus the promotion of desaturation of pre-existing fatty acids (Sato *et al.* 2003). A higher degree of membrane lipid fatty acid saturation could be a mechanism to maintain internal pH, since a higher degree of fatty acid saturation leads to lowered fluidity and lower CO_2-permeability of cell membranes (Rossoll *et al.* 2012).

Ocean acidification experiments generally aim to allow the prediction of the test organisms' future response to elevated pCO_2 A recent study by Tortensson *et al.* (2013) highlighted the need of conducting ocean acidification experiments under as close to natural conditions as possible, *i.e.*, nutrient concentrations, temperature, light intensities, *etc*. In the latter study the authors exposed the Antarctic diatom *Nitzschia lecointei* to 390 and 960 µatm CO_2 for 14 days under −1.8 and 2.5 °C temperature. They only found a significant difference in PUFA levels between the two CO_2 treatments at −1.8°C. Increased temperature affected the PUFA levels more drastically than

CO_2 concentrations. If this temperature dependency of the CO_2 response is universal for Antarctic phytoplankton, then this could explain why we saw very little significant differences in PUFA levels across our three species which were grown at approximately 2.9 °C. Furthermore, if the CO_2-induced response of phytoplankton biochemistry is greatly reduced under warmer temperatures, it is possible that a CO_2-signal was not detected under the noise of the data. Since ocean acidification experiments are often resource intensive, use of a large number of replicates is not always practical, which can make the detection of subtle differences difficult. While we performed every treatment in triplicate in our experiments, a larger number of replicates would improve our statistical power and ability to detect subtle differences between treatments. To facilitate increased replication the number of CO_2 treatments can be reduced in favor of more replicates per treatment. However, this reduces the resolution of a possible CO_2 dose-response-curve and the ability to detect any potential tipping points.

Except for EPA contents in *Gymnodinium sp.* and carbohydrate content in *Pyramimonas gelidicola*, we could not detect a CO_2 signal across all CO_2 concentrations, and often only found significantly different biochemical contents in the highest pCO_2 cultures compared to control cultures. As noted above, this could be due to noise in the data due to low replication, which only allows strong differences to be statistically significant. However, the chance of false positive results also increases with the number of parameters that are tested for. Increased replication and possibly reduced experimental temperatures are therefore recommended for future research.

5.5. Conclusion

In this study we have shown that an elevated CO_2 concentration has, at most, only modest effects on the biochemistry of three Antarctic phytoplankton species, although the responses were species-specific. It is unlikely that any phytoplankton species will be completely unaffected by changes in CO_2 concentration; however, the degree to which different species will be capable of tolerating ocean acidification, while simultaneously exposed to other climate-induced stressors, will determine which species will be the "winners and losers" in the future (Gervais and Riebesell 2001; Sobrino *et al.* 2008; Chen and Gao 2011; Arnold *et al.* 2013). While some of the species studied here showed responses in line with current literature, some of the results were contradictory. Laboratory experiments are not able to include all phytoplankton species of the oceans. Thus, to improve our ability to predict future changes of mixed phytoplankton communities, we need to increase our understanding of the underlying mechanisms by which pH and CO_2 availability affect phytoplankton physiology. An enhanced understanding will help explain the differences in species-specific responses and thereby improve our ability to extrapolate laboratory based results of individual species to natural communities. Our study suggests that increases in pCO_2 have the potential to alter the nutritional quality of individual phytoplankton species available for grazers via species-specific changes in their biochemistry, particularly the fatty acid profiles as emphasized by examination of the essential long-chain PUFA, and this adds to the importance of understanding how phytoplankton will change in a high-CO_2 ocean.

6. How rapidly is the biochemistry of the Southern Ocean diatom *Pseudo-nitzschia subcurvata* affected by a sudden change in pCO_2?

6.1. Abstract

Ocean acidification will impact phytoplankton on an individual species and / or community composition level. Laboratory ocean acidification experiments are an important tool to further our understanding on how phytoplankton will be affected. However, the experimental methodologies employed are still varied. In particular, there is no consensus on the need for or duration of acclimation periods prior to experiments, but it is now common practice to acclimate phytoplankton cultures for 7 – 9 generations. I conducted an experiment to establish how rapidly *Pseudo-nitzschia subcurvata*, a ubiquitous Southern Ocean diatom, can acclimate to a sudden change in pCO_2 and whether acclimation over several generations is necessary. This experiment also aimed to establish whether exposing the diatom for two days to a different pCO_2 environment during the krill feeding experiment described in Chapter 3 altered the algal biochemistry and thus potentially compromised the experimental design. Here I acclimated *Pseudo-nitzschia subcurvata* for at least 7 generations and then changed the pCO_2 environment suddenly; this was followed by regular sampling of the cultures for analysis of pigment and fatty acid composition, C:N ratio and carbohydrate concentration over the next 48h. An unequivocal interpretation of the results was hampered by an unintended depletion of nutrients during the course of the experiment but also by the fact that there was little difference in the measured biochemistry between high and low CO_2 cultures at the start of the experiment. With this in mind, I conclude that a rapid change in CO_2 concentration does not have as large an effect on the biochemistry of *Pseudo-nitzschia subcurvata* as does the depletion of nutrients. Based on these results I recommend incorporating

regular measurements of parameters such as nutrient concentrations and possibly also temperature throughout the experiment to assure that the responses seen are truly a reflection of the pCO_2 environment.

6.2. Introduction

While there is ample experimental evidence that phytoplankton species are affected by elevated levels of CO_2 (Riebesell *et al.* 2000; Lohbeck *et al.* 2012; Rossoll *et al.* 2012; Sugie and Yoshimura 2013), little is known about how fast phytoplankton cells adapt to a rapidly changing pCO_2 environment (Barcelos e Ramos *et al.* 2010; Mueller *et al.* 2010; Rossoll *et al.* 2012). Experiments with the well researched coccolithophore *Emiliania huxleyi* showed a similar physiological response in cultures exposed to an abrupt elevation in CO_2 concentrations over 26h as compared to cultures that had been acclimated over 7 – 9 generations (Barcelos e Ramos *et al.* 2010). In the same species Mueller *et al.* (2010) found the same changes in cultures acclimated to elevated pCO_2 over 152 generations as had been seen after 7 – 10 generations. Thus is it possible that phytoplankton cells are able to adapt very quickly to changes in their environment.

If the ability to adapt rapidly to changes in pCO_2 was common amongst phytoplankton species, it would validate experimental findings where an acclimation period was impractical (Tortell *et al.* 2002; Kim *et al.* 2006; Paulino *et al.* 2008; Riebesell *et al.* 2008). Therefore, I conducted an experiment to investigate how quickly *Pseudo-nitzschia subcurvata*, a ubiquitous Southern Ocean diatom, can acclimate to a rapid change in pCO_2 and whether acclimation over several generations is necessary. This experiment also aimed to establish whether exposing the diatom for two days to a different pCO_2 environment during the krill feeding experiment described

in Chapter 3 altered the algal biochemistry and thus potentially compromised the experimental design.

If *Pseudo-nitzschia subcurvata* can acclimate to the "new" CO_2 environment within 48h, i.e. it showed similar values for all biochemical parameters as the respective culture acclimated for at least 7 generations, then this would cast doubt on the validity of previous krill feeding experiments where phytoplankton cultures were transferred into krill jars of a different pCO_2 environment for two days (see Chapters 2 and 3).

To be able to determine a time point by which the phytoplankton cultures have acclimated to the new pCO_2 environment I needed measurements from acclimated cultures for comparison. The experimental design therefore included an acclimation period (at least 7 generations) and then a rapid change in the pCO_2 environment followed by regular sampling of the cultures for the next 48h. I chose 48h as timeframe since it was the duration used in the krill feeding experiment described in Chapter 3. This timeframe also compares to the experimental duration used by Barcelos e Ramos *et al.* (2010) and Rossoll *et al.* (2012). Culture samples were taken for analysis of pigment and fatty acid composition, carbon to nitrogen (C:N) ratio and carbohydrate concentration (CHO). Fatty acid analysis focussed on polyunsaturated fatty acids (PUFA), monounsaturated fatty acids (MUFA) and saturated fatty acids (SFA). These were chosen since the composition of PUFA and the ratio of PUFA to MUFA and SFA are critically important for grazer development, reproduction and hatch rates amongst other metabolic processes (Harrison *et*

al. 1990; Yoshida *et al.* 2011; Chen *et al.* 2012). C:N ratio is a bulk indicator of nutritional quality (Sterner *et al.* 1993) and pigment composition serves as an indicator of photophysiology.

6.3. Methods

6.3.1. Algal culturing

The basic structure of the continuous culture system, described in Chapter 4, was maintained for this experiment with one exception: while fresh media was supplied to each culture bag via a peristaltic pump as before, constant culture volume was maintained via a second peristaltic pump instead of passive overflow. Cultures were maintained in a temperature controlled refrigerator (average 3.6°C) in 4L custom – made, transparent plastic bags (polyethylene, Entapack, Australia). As this experiment was part of a larger experiment (Chapter III of the appendix), six replicate bags were grown per CO_2 treatment during the acclimation phase and combined into three replicates per treatment for the 48h experiment described here. To enable comparison with the semi – continuous culture experiment described in Chapter 3, the same irradiance level was used in this experiment. Cultures were exposed to continuous light intensities of 142 ± 20µmol m^{-2} s^{-1}, approximating moderate light levels (about 30% of levels observed on a cloudless day) at 5m depth (Thomson *et al.* 2008). Nutrient concentrations approximated those common for the source area for these strains (Davis Station, Antarctica), in modified f/2 medium (Guillard 1975). Nitrate (22µM ± 1.4) and phosphate (2µM ± 0.2) levels were adjusted to concentrations reported around O'Gorman Rocks, off

Davis Station, Antarctica (Gibson 1998; Roden *et al.* 2013). Silicate concentrations (27µM ± 2.1) were lower than those around O'Gorman Rocks due to seasonal changes of the nutrient levels in the seawater I used, but concentrations provided were not limiting (Harrison *et al.* 1977; Egge and Aksnes 1992). I chose f/2 medium as it lacks any buffer that could affect the carbonate chemistry and trace metal speciation in the growth medium and thereby affect medium pH and phytoplankton growth (Shi *et al.* 2009).

Cultures were bubbled continuously with filtered ambient air and CO_2 – enriched air, respectively for the two treatments. The cultures were allowed to acclimate to ambient and high CO_2 concentrations for at least 7 generations; three replicates at ambient pCO_2 (392µatm ±77) and three replicates at 878µatm ±148. After the acclimation period and prior to changing CO_2 concentrations, cultures were sampled for baseline results (t=0). Ambient cultures were then bubbled with CO_2 – enriched air of approximately 950ppm and high CO_2 cultures were bubbled with ambient air that had been passed through a column filled with soda lime to bind CO_2 before entering the cultures. This process took 2h to complete. Over the next 48h ambient cultures were maintained at 921µatm ±131 (low to high, LH treatment, 392µatm to 921µatm) and high CO_2 cultures at 391µatm ±64 (high to low, HL treatment, 878µatm to 391µatm). This way the average pH of ambient cultures was reduced from 8.07 (±0.044) to 7.72 (±0.066) and the average high CO_2 culture pH was raised from 7.72 (±0.012) to 8.06 (±0.064). Stable pCO_2 was maintained by continuously bubbling the cultures with CO_2 – enriched air and ambient air, respectively, over the 48h period.

Flow cytometry results indicated that the cultures were nutrient stressed after 24h, retrospectively confirmed by nutrient samples taken at t=4h from the filtrate of two cultures per treatment. Thus all cultures were halved and refilled at t=26h with fresh media acclimated to the correct CO_2 concentration.

6.3.2. Carbonate chemistry

CO_2 concentrations were calculated with the CO2SYS.BAS Excel programme (Lewis and Wallace 1998) based on total alkalinity, pH and temperature using the constants after Mehrbach *et al.* (1973) as refitted after Dickson and Millero (1987). pH was measured at every sampling time point with a Mettler Toledo Multi Seven pH meter (Mettler Toledo, Australia) calibrated to fresh *tris*– and aminopyridine artificial seawater buffer made according to the SOP 6a in "Guide to best practices for ocean CO_2 measurements" (Dickson *et al.* 2007). Alkalinity samples (50mL) were taken four hours after the start of the experiment, poisoned with 25µL saturated mercuric chloride solution and stored refrigerated in the dark until analysis in a closed cell on a Total Alkalinity Titrator ATT-05 (Kimoto, Japan). A temperature probe logged the air temperature inside the fridge every 5min.

6.3.3. Physiological and biochemical analyses

Cultures were filtered to provide samples for fatty acid, pigment and elemental composition, and particulate carbohydrate concentration at the start

of the experiment, t=0, and at regular intervals once CO_2 concentrations were adjusted, t=2h, 4h, 6h, 9h, 24h, 32h and 48h.

Cell abundance

Daily cell abundance samples were taken to monitor growth and relate biochemical measurements to a per cell basis. Cell abundance samples were analysed using a BD FACSCalibur cytometer (Becton Dickson, USA) equipped with a 488nm argon laser and cell concentrations were calculated by dividing event counts from bivariate scatter plots by the volume of culture analysed.

Pigments

Samples (~50mL by weight) were vacuum filtered onto 13mm GF/F filter (Whatman), blotted dry, placed in cryovials and immediately frozen in liquid N_2. The samples were stored at -135°C until further analysis using a modified method after Mock and Hoch (2005). Pigments were extracted with dimethylformamide and methanol, containing 140ng of apo-8'-carotenal (Fluka) internal standard and analysed by high pressure liquid chromatography (Zapata *et al.* 2000) using a Waters 626 pump, Gilson 233xL autoinjector, Waters Symmetry C8 column, a Waters 996 diode array detector and a Hitachi FT1000 fluorescence detector. Pigments were identified by comparison of their retention times and spectra with a sample of mixed standards from known cultures (Wright and Jeffrey 1997) injected at the start of each daily sample queue. Peaks were integrated using Waters Empower software, manually checked and corrected where necessary and quantified

using the internal standard method (Mantoura and Repeta 1997). Further details on the procedures are in Wright *et al.* (2010).

Cellular carbohydrate concentration and carbon to nitrogen ratio

Approximately 100mL (by weight) of culture was filtered onto muffled Quartz filter (Sartorius) and ¼ used for C:N analysis and the remaining ¾ for carbohydrate (CHO) analysis.

Phytoplankton particulate organic carbohydrates were first denatured into monosaccharides (adapted from Brown *et al.* 1998) and the carbohydrate concentration determined via the standard colorimetric method after Dubois *et al.* (1956) on a GBC UV-Vis 916 spectrophotometer (GBC Scientific Equipment, Australia).

Prior to C:N analysis, inorganic carbon was removed with 2M HCl. The C:N ratio was determined via a Thermo Finnigan EA 1112 Elemental Analyser (CE Instruments, UK).

Fatty acid analysis

Approximately 100mL (by weight) of culture was filtered on a pre – extracted (1:1 v/v chloroform : methanol) 25mm GF/F (Whatman) filter. Samples were trans – methylated to produce fatty acid methyl esters (FAME) by heating in methanol : chloroform : concentrated hydrochloric acid (10:1:1 v/v/v) at 80°C for 2 hours (Christie 1982). FAME were extracted into hexane : chloroform (4:1 v/v) and concentrated under a stream of nitrogen. An internal injection

standard (19:0 FAME) was added to each sample and the fatty acid composition analysed by gas chromatography (GC) using an Agilent Technologies 7890A GC (Palo Alto, California, USA) fitted with an Equity – 1 fused silica capillary column (15m x 0.1mm i.d., 0.1µm film thickness), an FID, a split/splitless injector and an Agilent Technologies 7683B Series auto sampler and injector. Helium was used as carrier gas. Peaks were quantified with Agilent Technologies ChemStation software (Palo Alto, California, USA) and individual components were identified using mass spectral data and comparison of retention time data with those obtained for authentic and laboratory standards. GC – mass spectrometric (GC-MS) analyses were performed on a Finnigan Thermoquest GCQ GC-mass spectrometer fitted with an on-column injector using Thermoquest Xcalibur software (Austin, Texas, USA) and a capillary column of similar polarity to that described above.

6.3.4. Statistical analysis

Biochemical results were analysed via classical multivariate analysis for repeated measures. Differences between acclimated cultures at t=0 were determined via analysis of variance (ANOVA). Significant differences were accepted at $p \leq 0.05$. Calculations were performed with the R software environment 2.14.2 (R Development Core Team 2012).

6.4. Results

The experiment and the results are divided into two components; the effects of sustained high CO_2 concentrations on the diatom *Pseudo-nitzschia subcurvata*, i.e. the results at time point t=0; and the effects of rapidly changing the pCO_2 environment on previously acclimated cultures, i.e. the results of all following time points t=2h to t=48h.

6.4.1. T=0, CO_2 effects on acclimated cultures

After acclimation to the two CO_2 treatments (392µatm ±77 and 878µatm ±148), there were few significant differences between the two groups and they were all found within the fatty acid profile (Table 6.1). High CO_2 concentrations did not significantly affect cellular CHO, C:N or pigment composition. However, PUFA comprised a higher percentage of total fatty acids in the high CO_2 cultures, predominantly due to the increased percentages of ω3 PUFA, particularly 20:5ω3 (eicosapentaeonic acid, EPA) ($p<0.05$). Acclimation to high CO_2 concentrations led to approximately 20% higher %EPA than acclimation to ambient CO_2 concentrations ($p<0.05$). The %PUFA of cultures acclimated to high CO_2 concentrations was approximately 10% higher at t=0h than those of cultures acclimated to ambient CO_2 concentrations ($p<0.05$). The %MUFA was slightly lower in cultures grown at high pCO_2 but this was not significant ($p=0.07$).

Table 6.1 Comparison of the biochemical composition of acclimated *Pseudo-nitzschia subcurvata* cultures at time point t=0 under low (392µatm) and high (878µatm) CO_2 concentrations, CHO = cellular carbohydrate concentration, PUFA = polyunsaturated fatty acids, MUFA = monounsaturated fatty acids, SFA = saturated fatty acids, EPA = eicosapentaenoic acid (20:5ω3), DHA = docosahexaenoic acid (22:6ω3), Chl a = chlorophyll a, Ddx = diadinoxanthin, dtx = diatoxanthin, *p<0.05

CO_2 treatment	**392µatm**	**878µatm**
CHO [pg/cell]	18 (±5.4)	14 (±3.5)
C:N	8.3 (±0.13)	8.0 (±0.53)
%SFA	16 (±7)	15 (±13)
%MUFA (p=0.07)	49 (±1)	45 (±3)
%PUFA*	35 (±2)	39 (±5)
%ω3*	22 (±2)	26 (±6)
%ω6	7 (±0.1)	7 (±0.8)
ω3 / ω6	3 (±0.2)	4 (±0.3)
%DHA	0.4 (±0.0)	0.5 (±0.2)
%EPA*	20 (±2)	23 (±1)
Total fatty acids [pg/cell]	9 (±1.8)	8 (±0.4)
Chl a [pg/cell]	1.2 (±0.2)	1.3 (±0.0)
(Ddx + dtx) / Chl a	0.28 (±0.03)	0.26 (±0.02)

6.4.2. T=2h to t=48h, the effects of rapidly changing pCO_2

This part of the experiment was designed to determine the speed of the response to changing pCO_2, but it was hampered by unexpected nutrient limitation. Nutrient concentrations during the acclimation phase were maintained by the constant replenishment within the continuous culture system. Once the cultures were taken out of the system and maintained in sterile glass bottles, nutrients were not replenished for the first 26h. Nutrient samples from CHO filtrates at t=4h revealed that the cultures had effectively utilized the available nutrients and thus nutrient concentrations were reduced to 0.09µM (±0.08) and 0.03µM (±0.02) nitrate, 0.09µM (±0.01) and 0.09µM (±0.01) phosphate and 1.34µM (±0.21) and 2.51µM (±0.18) silicate for LH (392µatm to 921µatm) and HL (878µatm to 391µatm) treatment respectively. These nutrient concentration results were not available during the experiment. Yet, flow cytometry data indicated that cultures were nutrient stressed at t=24h (data not shown) and thus all cultures were halved and replenished with fresh nutrients at approximately t=26h.

Carbon to nitrogen ratio

The C:N ratio changed over time in both treatments ($p<0.0005$), initially increasing (0-24h), then decreasing (24-48h), but this trend was not significantly different between treatments (Fig. 6.1). The overall C:N ratio was lower in HL cultures ($p<0.05$). There was no significant difference in C:N ratio at the start of the experiment, prior to the change in CO_2 concentrations,

but the ratio was significantly different at t= 24h and t= 48h ($p<0.05$). At both time points the C:N ratio of HL cultures was lower than LH cultures.

Carbon concentrations per cell were on average lower in the HL treatment, however, there was no significant trend with time for either treatment (Fig 6.2). Nitrogen concentrations per cell on average were not different between treatments.

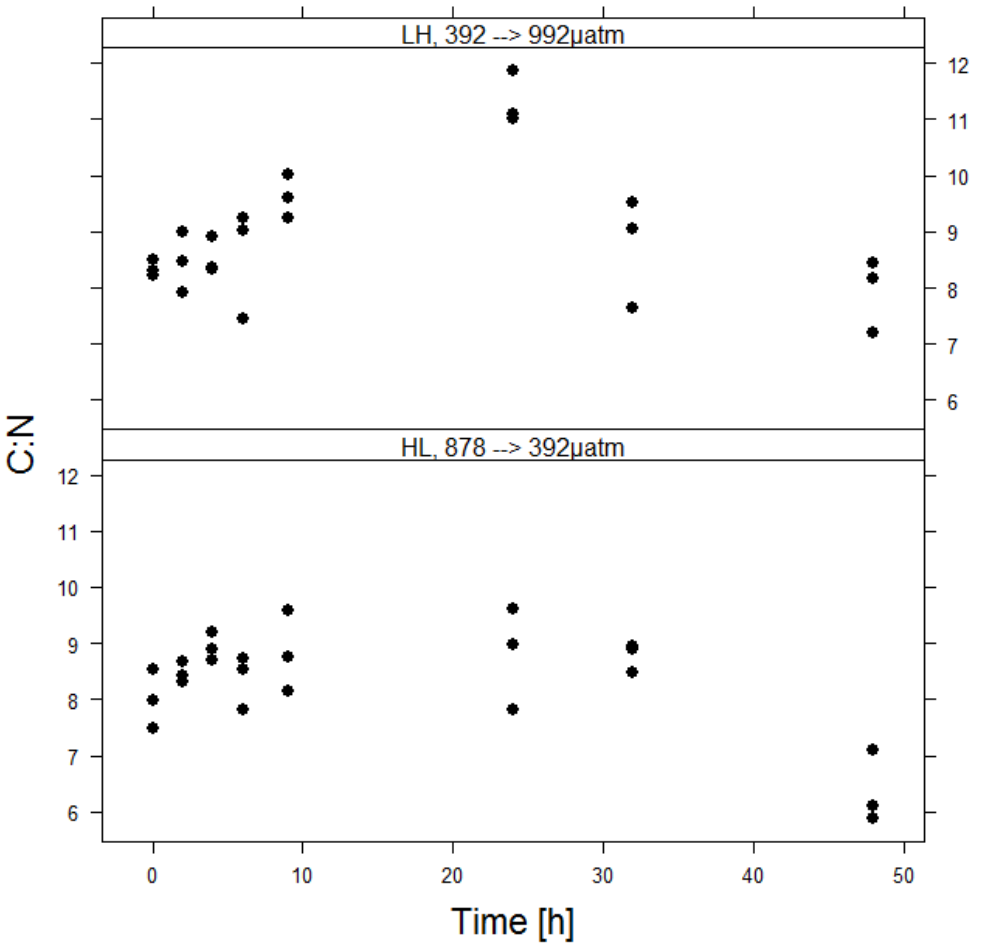


Figure 6.1 C:N ratio of *Pseudo-nitzschia subcurvata* cultures over time. The top panel represents the LH treatment, i.e. cultures grown at ambient CO_2 concentrations and then exposed to elevated pCO_2, while the bottom panel shows results from the HL treatment, i.e. cultures grown at elevated CO_2 concentrations and then exposed to ambient pCO_2.

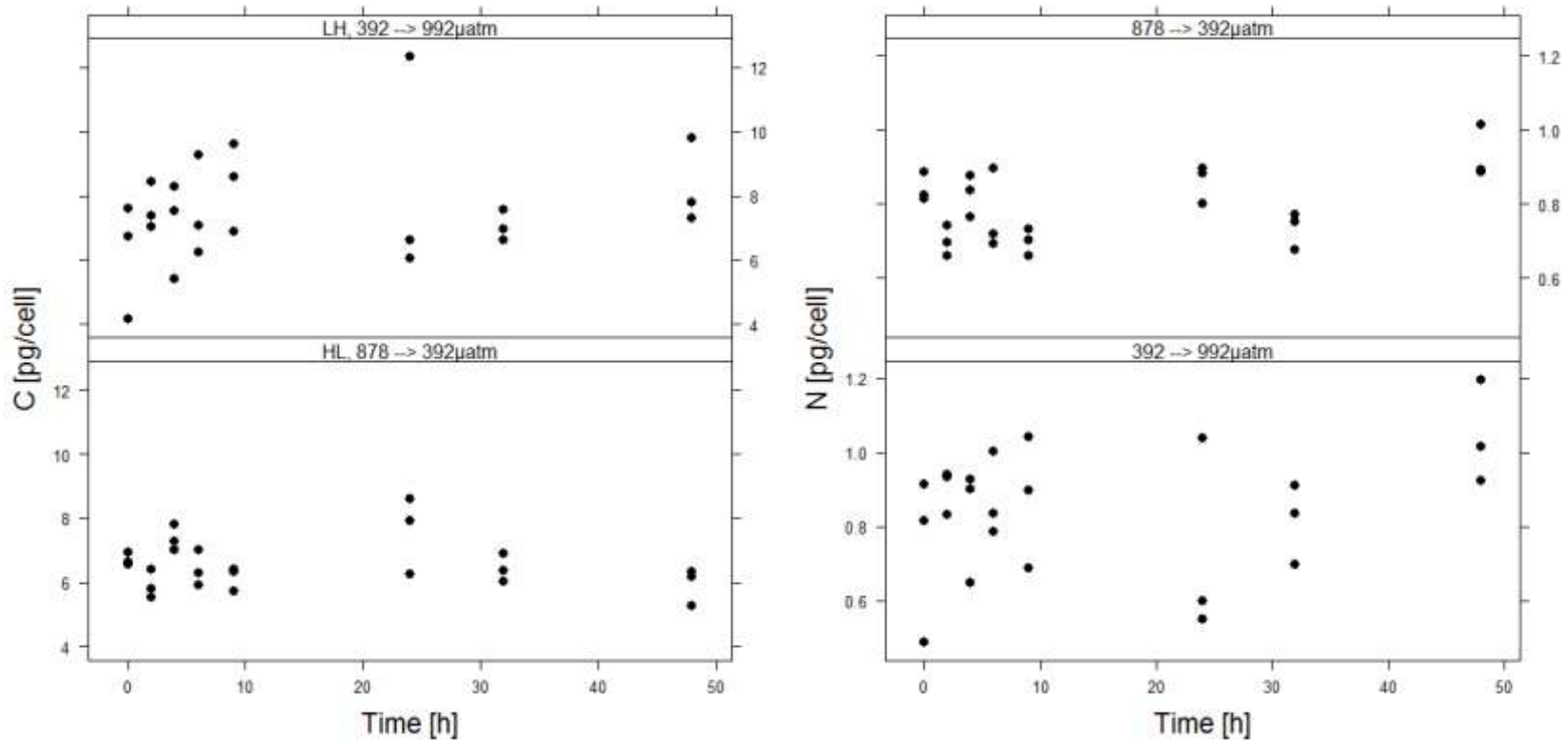


Figure 6.2 Carbon per cell [pg/cell] (left) and nitrogen per cell [pg/cell] (right) of *Pseudo-nitzschia subcurvata* cultures over time. The top panel represents the LH treatment, i.e. cultures grown at ambient CO_2 concentrations and then exposed to elevated pCO_2, while the bottom panel shows results from the HL treatment, i.e. cultures grown at elevated CO_2 concentrations and then exposed to ambient pCO_2.

Cellular carbohydrate concentration

Cellular CHO concentrations changed over time for both treatments (Fig. 6.3). Multivariate analysis showed a significant effect of time on CHO ($p<0.05$), however, the pattern of change over time was not significantly different between the two treatments. The linear increase in CHO levels during the first 9h of the experiment was significant for both treatments ($p<0.05$), but the decline from t=24h to t=48h was not ($p=0.07$) due to the large scatter in the LH treatment group. Individual simple linear regression showed a significant decrease in CHO for the HL cultures ($p<0.05$) but not for the LH cultures. There was weak evidence for overall lower CHO concentrations in HL ($p=0.064$).

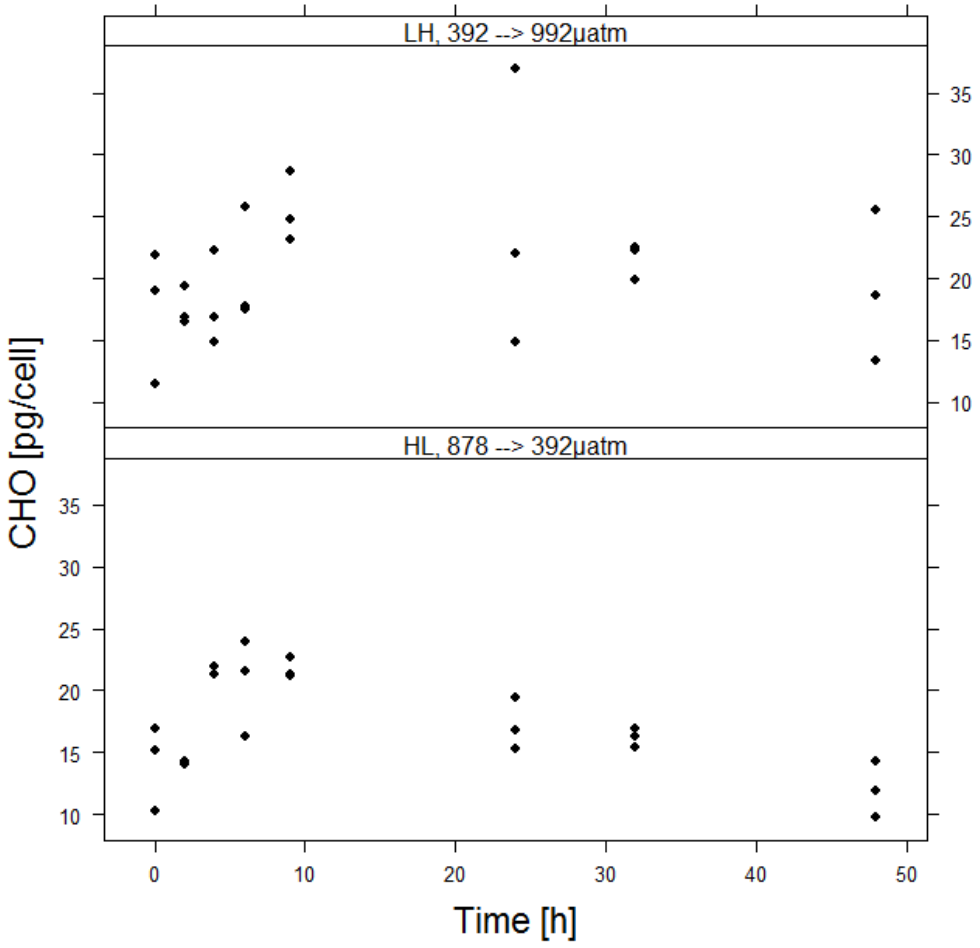


Figure 6.3 Cellular carbohydrate content [pg/cell] of *Pseudo-nitzschia subcurvata* cultures over time. The top panel represents the LH treatment, i.e. cultures grown at ambient CO_2 concentrations and then exposed to elevated pCO_2, while the bottom panel shows results from the HL treatment, i.e. cultures grown at elevated CO_2 concentrations and then exposed to ambient pCO_2.

Fatty acids

Total fatty acid concentration

Total fatty acids concentration per cell (TFA) decreased in LH cells between t=0 and t=9h ($p<0.05$), yet there was no significant trend for HL cells in the same time period. From t=24h to t=48h there was no significant change in either treatment, but TFA was overall marginally lower in HL cells ($p=0.055$), likely caused by the variation between replicates in the LH group (Fig 6.4).

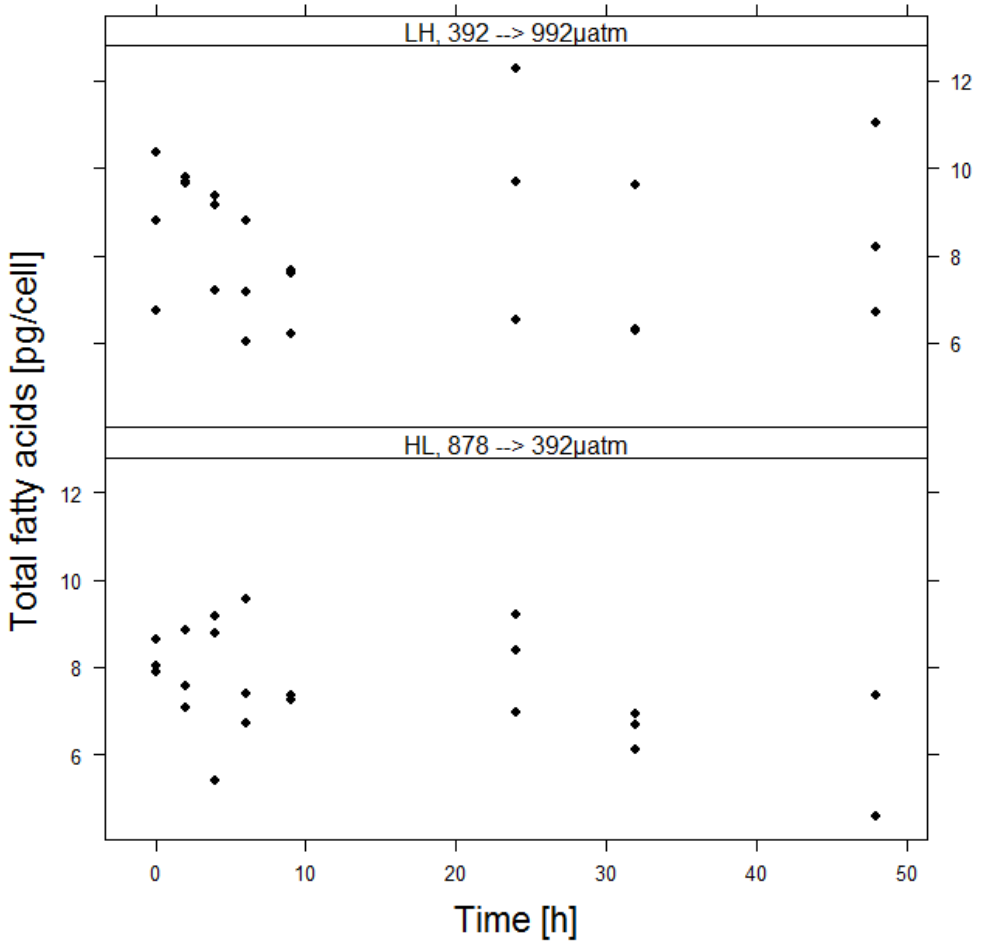


Figure 6.4 Total fatty acid concentration per cell [pg/cell] of *Pseudo-nitzschia subcurvata* cultures over time. The top panel represents the LH treatment, i.e. cultures grown at ambient CO_2 concentrations and then exposed to elevated pCO_2, while the bottom panel shows results from the HL treatment, i.e. cultures grown at elevated CO_2 concentrations and then exposed to ambient pCO_2.

Percentage of polyunsaturated fatty acids

Overall levels of %PUFA were lower in LH cells ($p<0.05$), but there was no significant change with time in %PUFA. Although %PUFA levels were lower in LH cells at the start of the experiment (10% at t=0 and 15% at t=2h), 48h after the change in CO_2 concentration %PUFA levels were no longer significantly different between the two CO_2 treatments.

Percentage of saturated and monounsaturated fatty acids

There were no differences in %SFA between treatments after acclimation to the two CO_2 environments, nor after CO_2 concentrations were rapidly changed. Overall %MUFA levels were significantly lower over the course of the 48h experiment in the HL cultures ($p<0.05$).

Percentage of omega 3 polyunsaturated fatty acids

The overall levels of %ω3 PUFA were significantly lower in LH cultures ($p<0.05$), however, %ω3 PUFA did not change significantly with time and by t=48h there was no difference between the two treatment groups.

Percentage of docosahexaeonic acid (22:6ω3, DHA) and eicosapentaenonic acid (20:5ω3, EPA)

Of the ω3 PUFA, the two health-benefitting long chain PUFA – DHA and EPA – are of particular interest. Acclimation to the two CO_2 concentrations did not affect %DHA, however once the CO_2 concentrations were changed, overall %DHA levels were lower in the LH cultures ($p<0.05$). There was a significant change in %DHA over time ($p<0.005$), with an initial increase (0 to 10h), then decrease (10h to 32h) and a further increase (23h to 48h). This trend was the same in both treatment groups (Fig. 6.5). Overall %EPA was lower in LH cultures ($p<0.01$), however, time did not affect %EPA in either treatment group (Fig. 6.5).

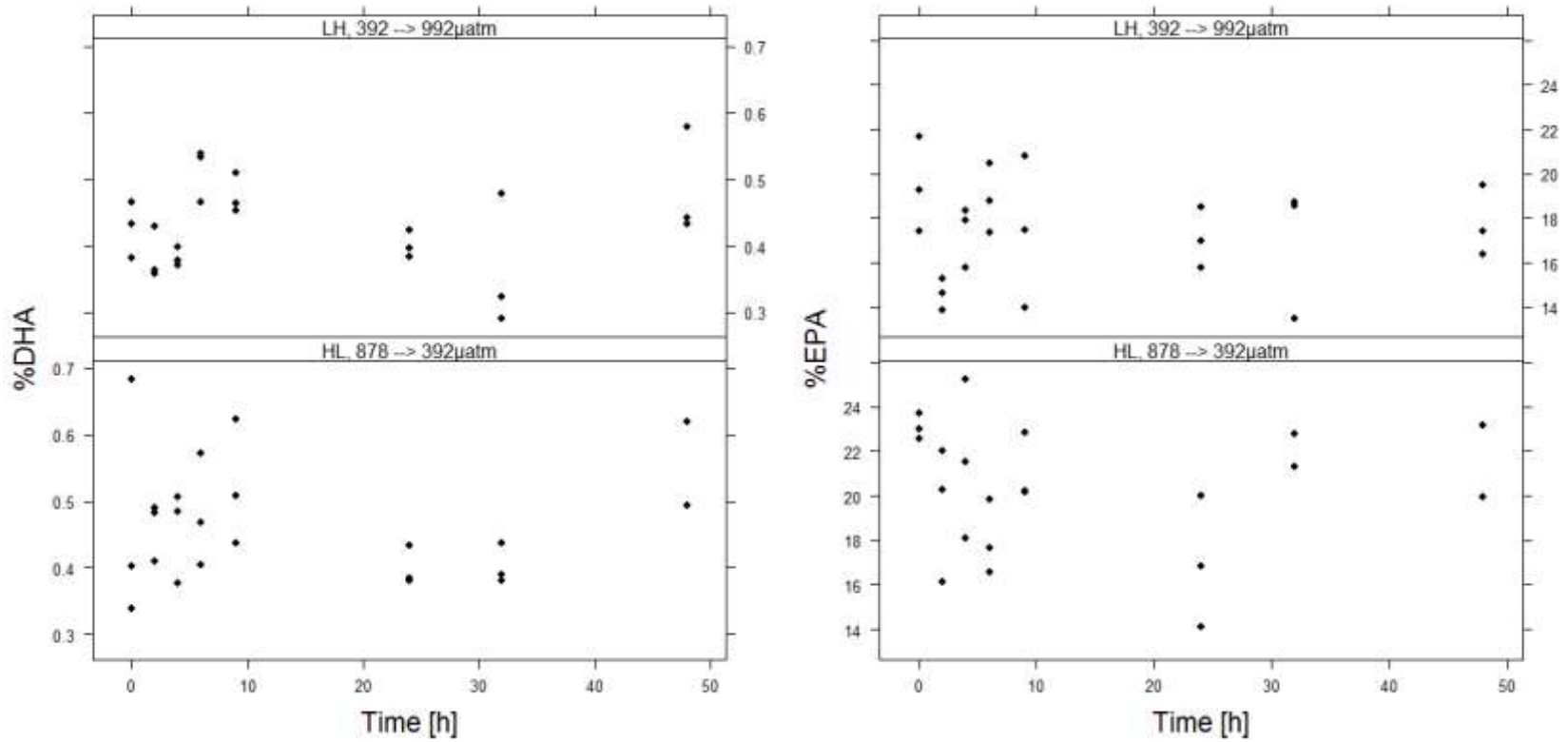


Figure 6.5 Percentage of 22:6ω3, DHA (left) and 20:5ω3, EPA (right) in *Pseudo-nitzschia subcurvata* cultures over time. The top panel represents the LH treatment, i.e. cultures grown at ambient CO_2 concentrations and then exposed to elevated pCO_2, while the bottom panel shows results from the HL treatment, i.e. cultures grown at elevated CO_2 concentrations and then exposed to ambient pCO_2.

No significant difference in DHA:EPA ratio was detected at the start of the experiment, however, over the course of the 48h DHA:EPA ratio was lower in the ambient to high CO_2 treatment group ($p<0.05$).

Percentage of omega 6 polyunsaturated fatty acids

There was an effect of time on %ω6 PUFA ($p<0.005$), with an initial increase (0 to 10h), a plateau (24h to 32h) and a final decrease (32h to 48h). The trend was not different between the two treatments ($p=0.653$). The overall levels were not different between groups and %ω6 PUFA were similar at the start of the experiment (Fig. 6.6).

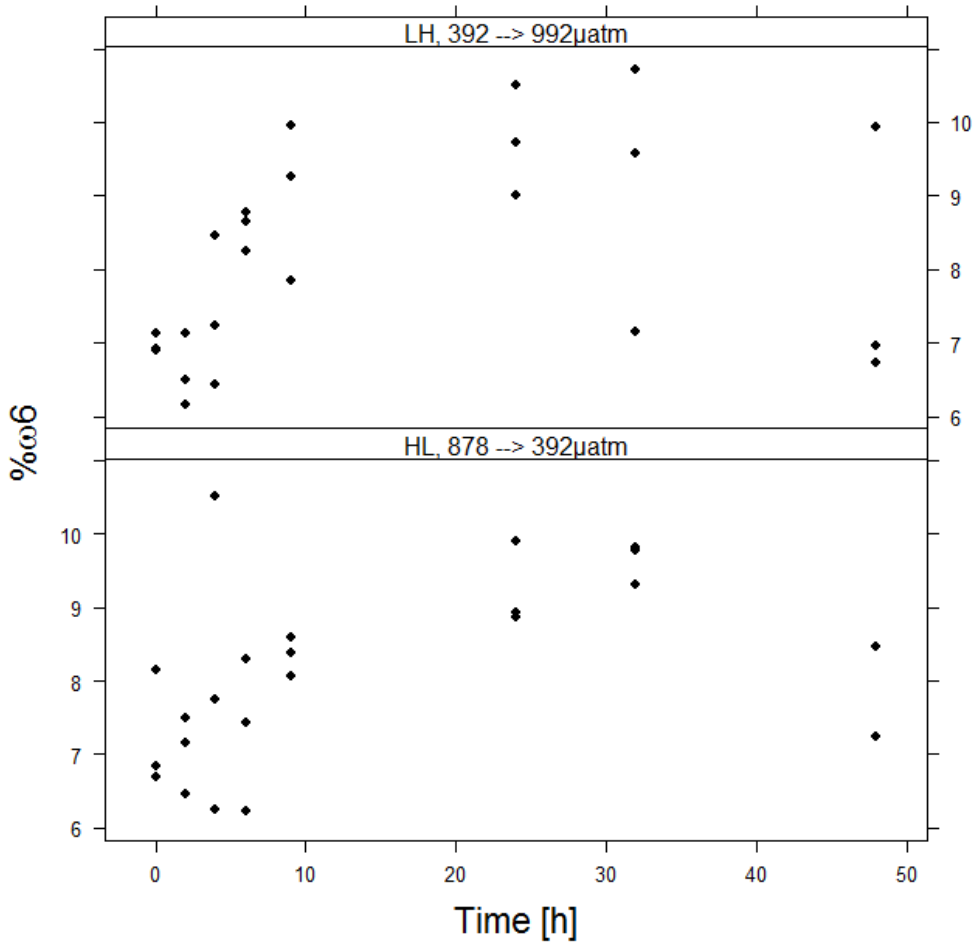


Figure 6.6 Percentage of omega 6 polyunsaturated fatty acids, %ω6, of *Pseudo-nitzschia subcurvata* cultures over time. The top panel represents the LH treatment, i.e. cultures grown at ambient CO_2 concentrations and then exposed to elevated pCO_2, while the bottom panel shows results from the HL treatment, i.e. cultures grown at elevated CO_2 concentrations and then exposed to ambient pCO_2.

Omega 3 to omega 3 polyunsaturated fatty acid ratio

The ω3:ω6 PUFA ratio was not affected by pCO_2 during acclimation. Once the CO_2 concentration was altered, ω3:ω6 PUFA ratio changed over time ($p<0.005$), but the change in pattern was similar in both treatment groups. The ω3/ω6 PUFA ratio was lower in LH cultures ($p<0.05$) (Fig. 6.8).

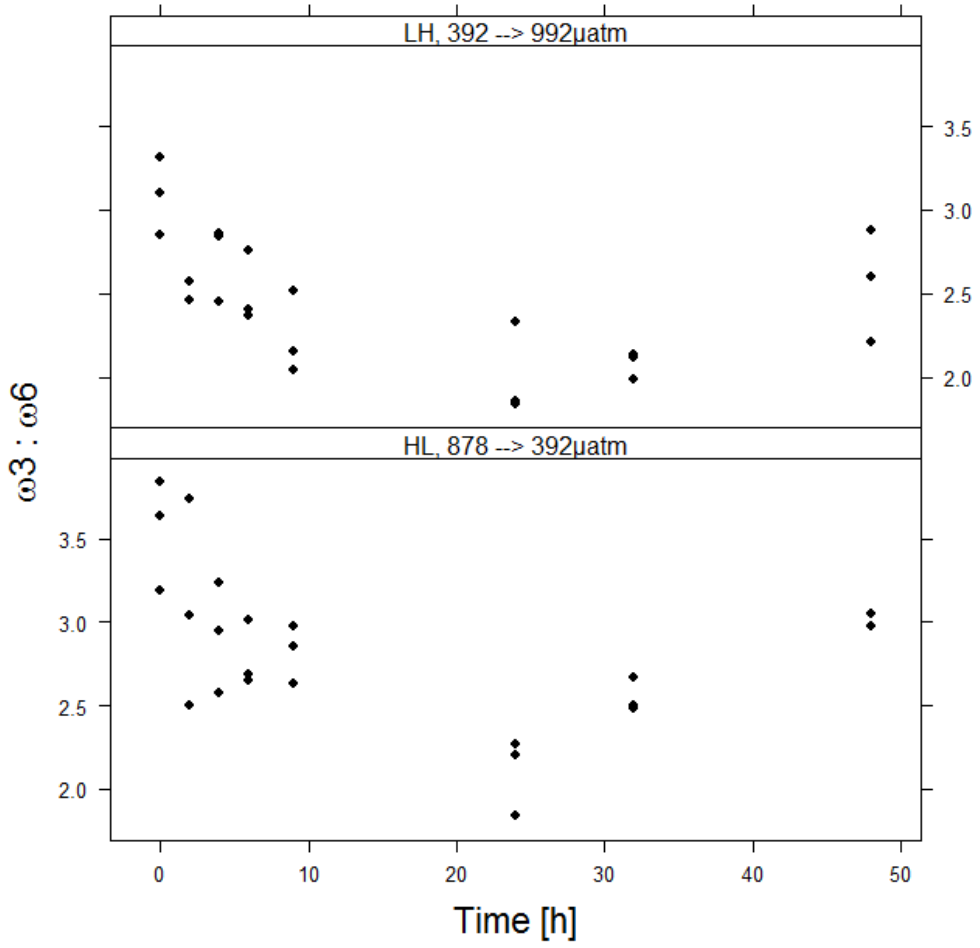


Figure 6.7 Omega 3 to omega 6 (ω3:ω6) PUFA ratio of *Pseudo-nitzschia subcurvata* cultures over time. The top panel represents the LH treatment, i.e. cultures grown at ambient CO_2 concentrations and then exposed to elevated pCO_2, while the bottom panel shows results from the HL treatment, i.e. cultures grown at elevated CO_2 concentrations and then exposed to ambient pCO_2.

Pigments

There were no significant changes in Chl a (chlorophyll a) concentrations per cell after the acclimation phase nor after CO_2 concentrations were changed. The ratio of the photoprotective pigments Ddx (diadinoxanthin) and dtx (diatoxanthin) to Chl a changed significantly over time once CO_2 concentrations were altered ($p<0.01$), resulting in a slightly lower ratio at 48h. The change was similar in both treatment groups and the overall levels were

not distinguishable between treatments (Fig. 6.8). While the ratio was approximately the same at the start of the experiment, (Ddx + dtx) : Chl a was significantly lower in HL cultures by t=48h ($p<0.05$).

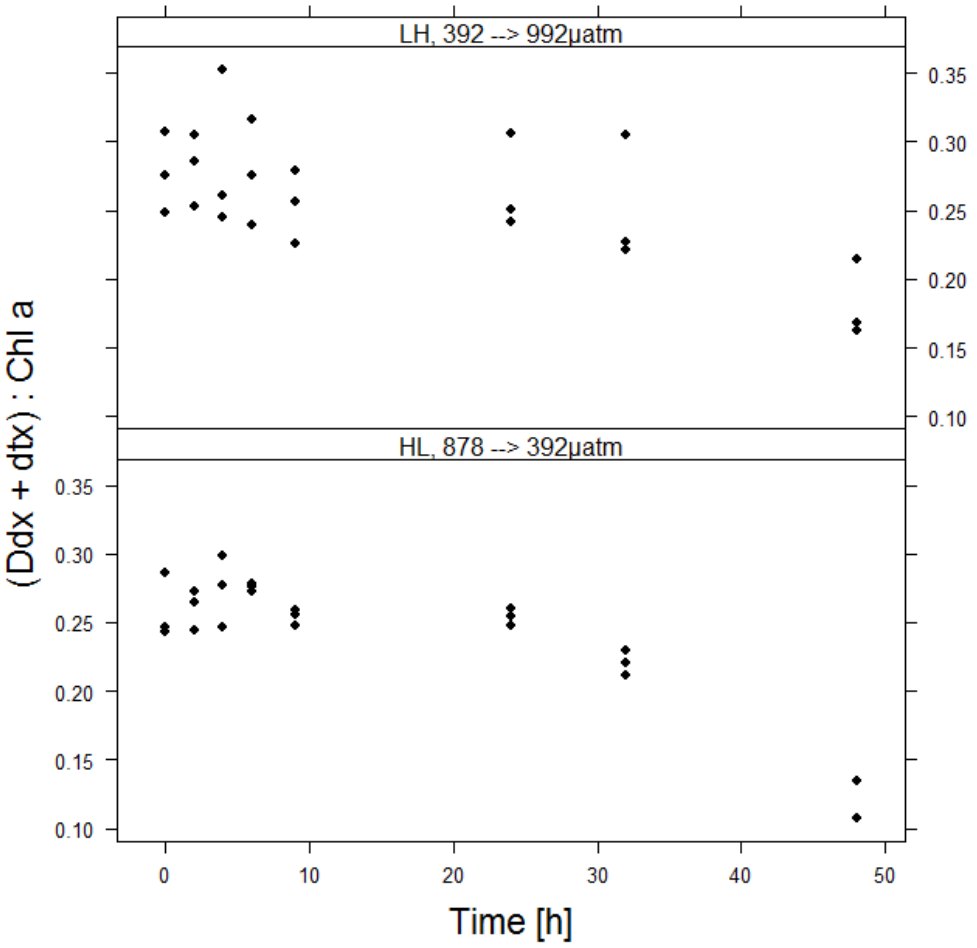


Figure 6.8 Sum of diadinoxanthin and diatoxanthin to chlorophyll a ratio (Ddx + dtx) : Chl a of *Pseudo-nitzschia subcurvata* cultures over time. The top panel represents the LH treatment, i.e. cultures grown at ambient CO_2 concentrations and then exposed to elevated pCO_2, while the bottom panel shows results from the HL treatment, i.e. cultures grown at elevated CO_2 concentrations and then exposed to ambient pCO_2.

6.5. Discussion

The overall aim of this experiment was to establish how quickly the Southern Ocean diatom *Pseudo-nitzschia subcurvata* can acclimate to a rapid change in pCO_2 and whether acclimation over several generations is necessary. This

experiment also aimed to establish whether exposing the diatom for two days to a different pCO_2 environment during the krill feeding experiment described in Chapter 3 altered the algal biochemistry and thus potentially compromised the experimental design. If rapid acclimation to changed carbonate chemistry was possible in *Pseudo-nitzschia subcurvata*, then the results of LH cultures (LH treatment, 392µatm to 921µatm) at the end of the experiment should be the same as of HL cultures (HL treatment, 878µatm to 391µatm) at the start of the experiment t=0, and vice versa.

6.5.1. CO_2 effects on acclimated cultures at time point t=0

Measurements from the start of the experiment at t=0 were taken to serve as the baseline for results obtained after CO_2 concentrations had been altered. Significant changes were only detected in the fatty acid profile and were opposite to previous findings with this species. In the fully replicated experiment described here, %PUFA increased, largely due to an increase in %EPA, while %MUFA decreased. During the semi – continuous batch culture experiment there was a decreasing trend in PUFA concentrations per cell and weak evidence for a decrease in %PUFA with increasing CO_2 concentration; the same applied to EPA. This difference in results emphasises the need for full replication, but it is also noteworthy that nutrient concentrations were similar, but not exactly the same during both experiments. Together with the difference in culturing methodology, semi – continuous (Chapter 3) *versus* continuous culturing (this study), the difference in nutrient concentration could account for the variation in results. Our findings are also

in contrast to reports of a decrease in %PUFA in the diatom *Thalassiosira pseudonana* at 915µatm (±270) CO_2 (Rossoll *et al.* 2012), but species specific responses to ocean acidification have been seen elsewhere (Burkhardt *et al.* 1999; Beardall and Raven 2004; Rost *et al.* 2008).

6.5.2. Effects of rapidly changing pCO_2 from time point t=0 to t=48hours

Rapid depletion of nutrients early into the 48h experiment was unexpected and compromised the interpretation of the results. Since nutrient levels were not recorded throughout the 48h for all replicates, it is an undefined factor that cannot be incorporated in the interpretation of the results. Yet it is a stressor that likely affected the parameters that were measured and thereby the results cannot be attributed unequivocally to either the changes in pCO_2 or nutrient concentrations. In the following discussion the results will therefore be interpreted with this in mind.

Rossoll *et al.* (2012) demonstrated that the fatty acid profile of the diatom *Thalassiosira pseudonana* can change within hours of exposure to a different CO_2 environment. Here I wanted to test whether this is the case for the diatom *Pseudo-nitzschia subcurvata*. For this, I acclimated the cultures to ambient and high CO_2 concentrations as the reference points prior to the 48h experiment.

Nutrient samples taken during the acclimation phase, while the continuous culture system was running, showed that nutrient concentrations were close to

target values (data not shown). However, during the 48h experiment, while the cultures were no longer connected to the continuous culturing system, nutrient concentrations were depleted rapidly. Therefore the experimental conditions during the 48h experiment were not comparable to those during the acclimation phase. For this reason the measurements taken from cultures exposed to high pCO_2 at t=48h cannot be compared to those taken from cultures acclimated to high pCO_2 at t=0 and I will only discuss the changes in phytoplankton response over time.

Carbon to nitrogen ratio

Despite the decrease in nutrient concentrations, nitrogen (N) per cell concentrations did not reflect signs of nutrient depletion. There was no significant increase in N per cell concentration after nutrients were replenished (t=26h) in either treatment, although there appeared to be an increasing trend in the LH cultures which was not significant due to the large variation on sampling point t=24h. The overall lower C:N ratio in HL cells was due to a lower carbon (C) per cell concentration and not due to a change in N per cell concentration between treatments. This could indicate that LH cells were able to utilize more CO_2 in the higher CO_2 concentrations compared to HL cells.

Overall the change in C:N ratio was not different between the two CO_2 treatment groups and this is likely due to the underlying nutrient limitation, as observed by Gervais *et al.* (2001) who reported a lack of CO_2 – induced effect on C:N:P on the diatom *Skeletonema costatum* under phosphorus limitation.

The increase in C:N ratio during the first 24h of the experiment could be due to a decrease in growth rates under nutrient limitation (Thomas and Dodson 1972), although this could not be confirmed here. Changes in growth rates within 24h could not be detected due to the slow division rate in this Antarctic species ($0.5 – 0.9 d^{-1}$ during previous high-CO_2 experiments, Chapter 3).

Cellular carbohydrate concentration

Both LH and HL cultures responded with an increase in CHO during the first 9h of the experiment. During this time nutrient concentrations were decreasing. By sampling point t=24h, CHO decreased back to starting concentrations. After this time point nutrients were replenished. While CHO of LH cells stabilized during the following 22h, CHO of HL cells declined significantly. Under nutrient limitation, C fixed by photosynthesis is incorporated into metabolites that do not contain N or phosphorus atoms (Shifrin and Chisholm 1981). An increase in CHO content under nutrient limitation has been reported in other studies (Shifrin and Chisholm 1981; Thomas *et al.* 1984; Harrison *et al.* 1990). This would explain the increase in CHO concentrations during the first 9h. Once nutrients had been replenished, CHO concentrations returned to values similar to those at t=0 in both cultures.

Fatty acids

Overall fatty acid profiles did not change markedly with the change in pCO_2. It is not possible to determine whether the initial decrease in total fatty acid (TFA) concentration in LH cultures was due to the change in pCO_2 or the gradual nutrient depletion. Based on nutrient samples from t=4h, nutrient

depletion occurred at a similar rate in both treatments. This would point towards the change in pCO_2 as cause for the initial decrease in TFA of LH cultures.

After acclimation to high CO_2 concentrations %PUFA was higher than in cells acclimated to ambient CO_2 concentrations and this relative difference remained on average unchanged even after CO_2 concentrations were altered. The same applied to %MUFA and %ω3 PUFA. The change in %DHA and ω3/ω6 PUFA ratio between t=0 and t=48h is interesting. In both treatments, %DHA levels increased during the first 9h of the experiment, but levels were close to start values by t=24h and slightly increased by t=48h. I hypothesise that this was due to the decrease in nutrient concentrations between t=0 and t=24h, after which nutrients were replenished. Possible secondary nutrient depletion could also explain why %DHA levels increased again by t=48h. Similar to CHO, fatty acids do not contain nitrogen or phosphorous and can thus be produced if photosynthesis is possible and nutrients are depleted (Shifrin and Chisholm 1981; Suen *et al.* 1987). Similarly ω3/ω6 PUFA ratio decreased between t=0 and t=24h and then increased between t=24h and t=48h in both treatments.

Lipid content is known to be influenced by environmental factors, particularly nutrient availability. However, the response to nutrient deficiency is not uniform across species. While some species show an increase in lipids and total fatty acids under nutrient limitation, others respond with a decrease and other species show no change in lipid content (Shifrin and Chisholm 1981;

Thomas *et al.* 1984; Taguchi *et al.* 1987; Harrison *et al.* 1990; Gong *et al.* 2013). It has been reported that phytoplankton can adapt to nutrient limitation not merely by increasing or decreasing the concentration of lipids, but also by changing the lipid class composition and ratio of fatty acids (Harrison *et al.* 1990). Under phosphorus limitation phytoplankton can substitute phospholipids with non – phosphorus lipids (van Mooy *et al.* 2009). It is therefore possible that the trend in %DHA was a result of changes in lipid composition caused by nutrient depletion.

Pigments

The ratio of photoprotective pigments to chlorophyll a (Chl a) decreased over time in both treatments. This could be due to a change in light intensities within the different culture vessels of acclimation phase and 48h experiment. Since this small 48h experiment was part of a larger experiment, described in Chapter III of the appendix, a subsample of the cultures was transferred from the plastic culture bags they had acclimated in to glass bottles for the 48h experiment. These bottles were then placed close to the plastic culture bags they were subsampled from. The glass bottles were larger in diameter than the plastic culture bags, so self – shading would have been higher in the glass bottles than the bags. The light intensity in the centre of the bottles could therefore have been lower and thus the need for photoprotective pigments would have decreased over time. This could explain the decrease in (Ddx + dtx) / Chl a ratio in both treatments. However, the decline in relative amounts of photoprotective pigments was stronger in HL cultures than LH cultures. Previous experiments described in Chapter 3 showed an increase in the

relative amounts of photoprotective pigments with increasing CO_2 concentrations and was attributed to a CO_2 – -induced photosensitivity. Under elevated CO_2 concentrations carbon concentrating mechanisms are likely down – regulated, which takes away a sink for excess energy. This mechanism could explain why (Ddx + dtx) / Chl a ratio decreased more in HL than in LH cultures.

6.6. Conclusion

In this experiment I wanted to resolve the question whether the diatom *Pseudo-nitzschia subcurvata* can adapt rapidly to changing pCO_2 conditions and whether exposing it to a different CO_2 environment for two days, such as during the krill feeding experiments (Chapters 2 and 3), has the potential to alter its biochemistry. An unequivocal interpretation of the results was hampered by the unexpected depletion of nutrients during the course of the experiment, and also by the fact that there was little difference in the measured biochemical parameters between high and low CO_2 cultures at the start of the experiment. With this in mind, the overall conclusion is that a rapid change in CO_2 concentration does not have as large an effect on the biochemistry of *Pseudo-nitzschia subcurvata* as the depletion of nutrients. This has been observed elsewhere (Gervais and Riebesell 2001; Riebesell *et al.* 2008). Yet, nutrient concentration and any changes therein are rarely monitored and / or reported (Crawfurd *et al.* 2011; Rossoll *et al.* 2012), despite the potential for nutrient concentration and / or nutrient speciation (Lefebvre *et al.* 2012; Sugie and Yoshimura 2013) to interact or mask CO_2 effects on phytoplankton physiology and biochemistry.

Since low nutrient concentrations or nutrient limitation is a real world scenario and will occur in combination with variations in light intensity, rising sea water temperature and increasing CO_2 concentrations, I strongly suggest monitoring or stabilizing nutrient concentrations throughout laboratory based ocean acidification experiments with phytoplankton, but also to investigate the synergistic mechanisms of abiotic factors on CO_2 – induced response of phytoplankton in future studies.

7. Conclusions and future directions

It is now common knowledge that ocean acidification has the potential to affect marine phytoplankton (Hein and Sand-Jensen 1997; Burkhardt *et al.* 2001; Engel *et al.* 2005; Millero *et al.* 2009; Mueller *et al.* 2010; Sugie and Yoshimura 2013), particularly phytoplankton biochemistry, i.e. C:N, carbohydrate concentration and fatty acid profile (Thornton 2009; Hoogstraten and Timmermans 2012; Rossoll *et al.* 2012; Schoo *et al.* 2013). It has also been shown that altered phytoplankton biochemistry can negatively affect development, growth and fecundity of grazers (Kilham *et al.* 1997; Urabe *et al.* 2003; Urabe and Waki 2009; Rossoll *et al.* 2012; Schoo *et al.* 2013). Therefore the fundamental question for this research project was, whether ocean acidification has the potential to affect the key species of the Antarctic food web, *Euphausia superba*, through impacts on phytoplankton nutritional quality. In Chapter 1, I outlined a number of questions that need to be answered to achieve this goal and I designed and conducted five experiments to address three questions:

a) Are Antarctic phytoplankton species susceptible to ocean acidification?
b) If so, in what way will ocean acidification affect the biochemistry of Antarctic phytoplankton?
c) Can these changes in individual phytoplankton species negatively affect grazing Antarctic krill?

Since a significant portion of this research project was dedicated to the development and construction of experimental facilities, the particular challenges and suggestions for improvements of the different experimental

setups will be discussed first. Then the answers to the above outlined questions will be considered alongside the broader questions of ecosystem level responses to acidification which were beyond the experimental scope of this study.

7.1. Challenges of laboratory based ocean acidification experiments

7.1.1. Special considerations for ocean acidification experiments with phytoplankton

Conducting ocean acidification experiments with phytoplankton in the laboratory presents a number of challenges. CO_2 concentrations have to be kept stable throughout the experiment. Phytoplankton cells need to be kept in exponential growth and light intensity and nutrient concentrations should approximate those found in the natural environment of the species investigated, since physiology and biochemistry can be influenced by the interaction of environmental conditions and CO_2 concentration.

In order to conduct ocean acidification experiments, experimental facilities at Australian Antarctic Division laboratories had to be designed and constructed. I conducted extensive developmental work and undertook a series of pilot studies, using Southern Ocean phytoplankton. I found using an automated, continuous culturing system was the best approach. This system was less labour intensive than a semi – continuous culture system and eliminated the need to manually maintain the CO_2 levels within the culture vessels. In the continuous culture system overflowing culture was available for biochemical

analyses and this allowed a reduction in size of each culture vessel. Replication of each treatment in the continuous culture system was thereby also possible and full replication greatly improved the statistical power to detect significant changes between treatments.

To avoid disturbances to the culture physiology by removing excess cell numbers, I chose to sample from the excess culture of the continuous culture system, collected in overflow bottles. While CO_2, temperature and light conditions were the same as within the continuous system, nutrients were not renewed during the time the culture was left to accumulate within the overflow bottles. However, due to the slow doubling time (~ 2d), those cells entering the overflow bottle at the start of the two day period would have undergone a maximum of one generation outside of the continuous culture system *versus* at least five generations of acclimation within the continuous culture system. Further, cell densities within the overflow bottles never exceeded the carrying capacity associated with our culture medium, as established prior to the experiments. I therefore assumed that samples taken from the overflow bottles were representative of the culture within the continuous system.

To obtain a comprehensive picture of how pCO_2 affects phytoplankton physiology and biochemistry and therefore nutritional quality, a range of parameters are needed: carbohydrate concentration, lipid content and fatty acid profile, C:N ratio and ideally also carbon to phosphorus ratio and dissolved organic carbon excreted by the cells. All these parameters require a

substantial amount of culture volume for analyses, particularly if cell densities are kept low by the necessary low nutrient concentrations and / or to minimize major changes in culture pCO_2 by photosynthesis. To avoid the issues associated with sampling from excess culture accumulating in an overflow bottle, the culture vessels would need to be 10 times the total volume required for all analyses to keep culture removal below 10% of the culture volume. Given the number of analyses, this could easily amount to more than 10L of culture. At that size of culture volume, replication will become impractical, thereby compromising the statistical quality of the data. A compromise could be to limit the number of analyses at the expense of the completeness of the results.

Alternatively, a faster growing phytoplankton species could be chosen, which would require faster dilution rates of the culture vessel in a continuous culture system. Sample culture would accumulate in the overflow bottle much faster, thereby minimizing the time the culture remains outside the stable environment of the continuous culture system. The use of easy to maintain and well researched model organisms for experimental work is a common occurrence, although of questionable value for extrapolation of results into the natural environment.

Finally, sampling for the various analyses could be done on consecutive days from within the continuous culture system. This way removal of cells is kept minimal while still maintaining the same suite of measurements. The working hypothesis of a continuous culture system is that once the culture is

acclimated to the experimental conditions cell physiology and biochemistry should remain constant throughout the experiment, as long as the experimental conditions, and growth rates are kept constant. However, it is also well known that minor changes in growth rate and / or diurnal variations can affect cell physiology and biochemistry, so it is preferable to take all samples from the same point in time during the experiment to be able to relate all results to each other.

7.1.2. Special considerations for feeding experiments with Antarctic krill

In the course of the feeding experiments with Antarctic phytoplankton and krill a number of challenges were identified:

The food supplied to krill had to be renewed frequently. This was to avoid starvation and thereby compromising the dietary effect of the experimental food, and also to minimize bacterial growth on organic matter and faecal pellets. For sufficient replication I needed a large number of krill jars and thus preparation of fresh food was labour intensive.

Transferring the animals from one experimental vessel into the newly prepared one represented considerable handling stress. Also of concern was the possibility of losing krill, particularly the first larval stages which are very small.

Finally, in both krill experiments the DHA:EPA ratio decreased and %C, C:N ratio and ω6 PUFA levels increased in krill larvae feeding on high – CO_2 grown algae. This result was not directly reflected by similar changes in the phytoplankton biochemistry. This raises the question whether there were underlying changes in the phytoplankton cells that were not measured, such as variations in the cell morphology. These variations could affect palatability and subsequently feeding rate which would influence larval biochemistry. Or perhaps these results could have been a consequence of the experimental design. In both experiments phytoplankton cells were taken out of the growth vessel, and subjected to a different CO_2 level in the krill jar. Furthermore, cultures were diluted with sterile seawater to varying degrees to maintain stable cell densities across all krill jars. This meant that for the following two days high CO_2 grown phytoplankton cells were exposed to a different CO_2 concentration than they were acclimated to, with gradually changing nutrient concentrations. Although this was the case for all four CO_2 treatments, I cannot rule out that these changes interacted with the CO_2 – induced variations in the phytoplankton biochemistry and caused the observed trend within the krill larvae. Therefore a short term experiment was conducted to test this. Results from a 48h experiment using the diatom *Pseudo-nitzschia subcurvata* showed biochemical parameters such as cellular carbohydrate and total fatty acid concentrations changed within hours of exposure to diminishing nutrient levels, yet very little effect was seen due to a change in pCO_2.

7.2. Antarctic phytoplankton response to elevated pCO_2

Four experiments using five Antarctic phytoplankton species found that these species were susceptible to ocean acidification, however, the response was species – specific and in some cases subtle when compared to the magnitude of change caused by nutrient limitations. This is consistent with the range of responses of phytoplankton to elevated pCO_2 treatments reported in the literature.

In this study cellular carbohydrate concentrations in phytoplankton often increased under increased CO_2 concentrations. Increases in cellular carbohydrate concentration, as seen in the high CO_2 treatments, have been reported in other studies on phytoplankton under nutrient limitation (Shifrin and Chisholm 1981; Thomas *et al.* 1984; Harrison *et al.* 1990), where C fixed by photosynthesis is incorporated into metabolites that do not contain N or P atoms (Shifrin and Chisholm 1981). Accumulating carbon in the form of carbohydrates or excreting it as extracellular polysaccharides could therefore be a mechanism to deal with the excess carbon available to phytoplankton cells in a high CO_2 environment.

With an excess of carbon available to the phytoplankton, I predicted to find an increase in C:N ratio in the phytoplankton cells of the elevated pCO_2 treatments, particularly at the low nutrient concentrations provided. This working hypothesis was based on literature reports of enhanced carbon absorption and carbon production (Riebesell 2004; Riebesell *et al.* 2007;

Bellerby *et al.* 2008), even though these findings were from natural phytoplankton communities and can be partially explained by changes in the species composition. No significant changes in C:N ratio were found in any of the five phytoplankton species studied. This agrees with the minor changes in C:N ratio at above ambient CO_2 concentrations found by Burkhardt *et al.* (1999) in seven phytoplankton species, although it is in contrast to the rise in C:N under elevated pCO_2 in the cryptophyte *Rhodomonas salina* (Schoo *et al.* 2013) and the Southern Ocean diatom *Proboscia alata* (Hoogstraten and Timmermans 2012). We used nutrient concentrations approximating those reported for the area close to where our phytoplankton strains were isolated. This is in contrast to other studies which used nutrient enriched culture media such as f/2 (Burkhardt *et al.* 1999; Berge *et al.* 2010). Gervais *et al.* (2001) did not detect any effect of CO_2 concentration on C:N under phosphorous limitation. This could explain why we found very little evidence for CO_2 – induced changes in elemental composition at the low nutrient concentrations used. It is unclear why an increase in cellular carbohydrate concentration did not translate into higher C:N ratios, particularly since lipid content per cell did not decrease.

The changes in fatty acid profile were of particular interest in this study, as an adequate amount and the right ratio of essential fatty acids are important to Antarctic krill (Yoshida *et al.* 2011). Polyunsaturated fatty acids often decreased with increasing CO_2 concentration and this was also reported by Rossoll *et al.* (2012). Changes in the levels of the important ω3 long-chain PUFA, DHA and EPA, however, were less predictable. Detailed studies of the

mechanisms and pathways of lipid and fatty acid production in other organisms suggest that external and internal pH influence lipid and fatty acid production. A decrease in external pH can translate into a decrease of internal pH (Lane and Burris 1981), which in turn can suppress phospholipid metabolic genes, as reported in yeasts (Young *et al.* 2010). A lower degree of unsaturation of fatty acids in CO_2 – enriched cultures of *Chlorella kessleri* compared to ambient CO_2 concentrations was at least partially attributed to suppressed fatty acid synthesis and thus the promotion of desaturation of pre – existing fatty acids (Sato *et al.* 2003). A higher degree of membrane lipid fatty acid saturation could be a mechanism to maintain internal pH, since a higher degree of fatty acid saturation leads to lowered fluidity and lower CO_2 – permeability of cell membranes (Rossoll *et al.* 2012).

The CO_2 – induced variations in cellular carbohydrate concentrations (between -22 and +33% of control levels) observed during the four experiments are comparable to literature reports (+23; -22%, Taraldsvik and Myklestad 2000; Thornton 2009). However, the gradual depletion of nutrients experienced during the short – term 48h experiment described in Chapter 6 caused a 46 – 54% increase in cellular carbohydrate concentration within 9h. Abiotic factors such as nutrient concentration and / or light levels and diurnal variations could likely be a larger driver of biochemical change within phytoplankton cells than CO_2 concentrations (Burkhardt *et al.* 1999; Gervais and Riebesell 2001; Riebesell *et al.* 2008). This highlights the need to investigate CO_2 effects on phytoplankton cells under conditions that are

representative of the natural environment, i.e. light levels, nutrient concentrations and temperature.

7.3. Antarctic krill response to phytoplankton cells grown at elevated pCO_2

To determine whether these changes in individual phytoplankton species negatively affect grazing Antarctic krill, I conducted three experiments with Antarctic krill larvae and juveniles, feeding on phytoplankton grown at elevated pCO_2. These experiments showed that larval krill mortality can increase due to diet, although the observed changes in the phytoplankton biochemistry were not directly reflected in krill biochemistry.

Evidence that changes in phytoplankton cells can influence the next trophic level, i.e. grazers, was provided by observed increases in the daily mortality rates of larvae fed on *Pseudo-nitzschia subcurvata* culture grown under elevated CO_2 concentration. This increase in daily mortality rates in larval krill was attributed to a decrease in polyunsaturated fatty acids in the phytoplankton cells grown at elevated pCO_2. It cannot be ruled out that changes in the cell morphology, such as cell wall thickness or tendency towards chain formation as influenced by pCO_2 was also a contributing factor and these parameters should be investigated in future research.

Overall my findings are consistent with literature reports that high pCO_2 reduces the nutritional quality of phytoplankton for egg production rates, growth and larval survival of grazers (Urabe and Sterner 1996; Urabe *et al.*

2002; Urabe *et al.* 2003; Rossoll *et al.* 2012). It is difficult to estimate the relative importance of the direct effects of ocean acidification on Antarctic krill *versus* indirect effects via the altered nutritional quality of phytoplankton. Kawaguchi *et al.* (2013) found strong direct effects of increased seawater pCO_2 on hatch rates; an elevation of seawater pCO_2 to 1750µatm reduced hatch rates to 40% of control levels. In our study, a change in the biochemistry of *Pseudo-nitzschia subcurvata* increased larval mortality rates from 30% to 48%. This is a moderate increase in larval mortality compared to the reduction in hatch rates due to ocean acidification directly, however, a small increase in larval mortality in the laboratory can have much larger impacts in the natural population where other stressor might exacerbate the negative effect. Findings by Yoshida *et al.* (2011) reported a reduction in hatching success from 24.3% to 7% in females who were fed a diet insufficient (based on fatty acid profiles). These findings suggest that krill embryonic development is strongly influenced by the diet of spawning females. Species – specific differences in the phytoplankton response (Burkhardt *et al.* 1999; Mueller *et al.* 2010; McCarthy *et al.* 2012) and in the level of susceptibility of the grazer, make it difficult to extrapolate single – species experiments to whole ecosystems. Therefore, to improve our ability to extrapolate laboratory based results into the field, mixed phytoplankton species experiments with Antarctic krill larvae and adults, as well as combined experiments which investigate the impact of ocean acidification and altered phytoplankton biochemistry together are highly recommended.

7.4. Conclusions, wider implications and future research directions

In conclusion, we designed a relatively inexpensive continuous culture system that was able to maintain stable CO_2 concentrations within the phytoplankton cultures for long-term experiments. Through the course of four experiments with five Antarctic phytoplankton species, I found that phytoplankton respond in a species – specific manner to elevated pCO_2. Further, a short – term experiment showed that changes in abiotic factors such as nutrient concentrations can have larger effects on phytoplankton biochemistry than rapid changes in CO_2 concentrations. Finally, a feeding experiment with Antarctic krill larvae showed that CO_2 – induced changes in phytoplankton biochemistry, or potentially other characteristics such as morphology, can affect larval mortality rates. The exact mechanisms of this effect could not be established, and this finding warrants future research into the impacts of ocean acidification on phytoplankton as food source for Antarctic krill.

In this research project I conducted single – species phytoplankton experiments. Yet, to predict the effects of ocean acidification on phytoplankton as food source for krill, we also need to investigate how both individual species and the whole phytoplankton community will change under the impacts of ocean acidification. Questions posed for further research include:

a) Will Antarctic phytoplankton community structure change due to ocean acidification?
b) How will potential changes in the biochemistry of individual phytoplankton species together with shifts in phytoplankton community composition affect the nutritional quality of the whole phytoplankton community as food for krill?
c) Do Antarctic krill have the ability to detect changes in their food and adapt their diet if necessary?

Numerous experiments exposing natural phytoplankton communities to elevated pCO_2 have reported shifts in the community composition due to the effects of ocean acidification (Tortell *et al.* 2002; Feng *et al.* 2010; Leu *et al.* 2013). Phytoplankton species are not equal in their nutritional quality as food for grazers (Jonasdottir and Kiorboe 1996; Ban *et al.* 1997), so any shifts in the community composition may improve or deteriorate the overall nutritional quality. Shifts in phytoplankton community C:N:P due to ocean acidification have been shown by Bellerby *et al.* (2008). Yet Leu *et al.* (2013) concluded that despite the CO_2 – induced shift in the species composition of a natural Arctic community, the overall nutritional quality was not negatively affected, as measured by the composition of essential fatty acids.

Grazers such as Antarctic krill feed on a number of species. It is therefore possible that the mix of food species consumed will mitigate any negative CO_2 – induced changes in the biochemistry of individual phytoplankton species, as has been found for mixed diets and in natural communities with

microzooplankton (Suffrian *et al.* 2008; Urabe and Waki 2009; Aberle *et al.* 2013). Future experiments should investigate the possibility of selective grazing by Antarctic krill. Krill's ability to feed selectively on phytoplankton of equal size has been shown by Habermann *et al.* (2003) and this could be a possible mitigation strategy for krill in mixed phytoplankton communities of variable nutritional quality.

Experiments are by necessity a simplification of a complex natural system into one that can be artificially recreated, controlled and manipulated in the laboratory. Therefore, findings from these experimental systems can only be extrapolated into the natural world with caution. In nature, phytoplankton will not only be exposed to elevated pCO_2 and lowered pH, but also to increased sea surface temperatures, changed nutrient concentrations and ultraviolet radiation, amongst other factors. There are a large number of scientific publications that clearly point out how phytoplankton can be affected by each one, and a combination of, these abiotic factors (Sobrino *et al.* 2008; Beardall *et al.* 2009; Feng *et al.* 2009; Chen *et al.* 2012). For future research there is an urgent need for experiments that focus on the combined effects of the environmental factors that will be affected by climate change and investigate whether they will be acting synergistically or antagonistically.

The findings of this research provide a valuable step in assessing whether the basis of the Antarctic food web could be threatened by ocean acidification via more than just the direct pathway of increased seawater pCO_2 and lowered pH. Based on the findings of this research project I conclude that elevated CO_2

affects Southern Ocean phytoplankton biochemistry, albeit in a species – specific manner. There is also evidence that the effects of elevated pCO_2 on phytoplankton can be transferred to the next trophic level, in this case Antarctic krill larvae. However, the precise mechanisms by which CO_2 caused the observed changes in phytoplankton biochemistry and the flow – on effects on larval mortality require further investigation. Specifically, the interactive effects of nutrient concentration and CO_2 levels on phytoplankton need further research.

Appendix

I. Development of a semi – continuous culture system for ocean acidification experiments with phytoplankton and Antarctic krill

a. Preliminary setup for high – CO_2 phytoplankton experiments

As a preliminary trial for manipulating seawater pCO_2, I tested introducing CO_2 by continuously flushing the headspace of a culture vessel with 0.2µm filtered CO_2 – enriched air in preliminary experiments. Figure 1 shows the stability of pH in a full strength K medium (Keller *et al.* 1987), without the usual addition of tris (tris-(hydroxymethyl)-aminomethane) buffer. The medium was inoculated with the diatom *Synedropsis hyperborea*. Filtered air (0.2µm) was enriched with CO_2 gas to achieve a CO_2 concentration of around 1000ppm. The resulting CO_2-air mix was flushed into the headspace of 750ml culture vessels placed in a cold room on a shaker table (Fig. 2). The shaker table helped facilitate the equilibration of CO_2 concentrations between headspace and culture medium by reduction of boundary layers. Lights were set to a 12 / 12 hour on / off cycle and diurnal effects of phytoplankton physiology became noticeable from day seven, when the cell density was high enough to affect CO_2 concentrations noticeably. When the lights were turned off, photosynthesis ceased and the CO_2 produced by respiration resulted in a decreased pH. Once the lights were turned on again, photosynthesis absorbed CO_2, outweighing the production of CO_2 by photorespiration and thereby increasing pH again.

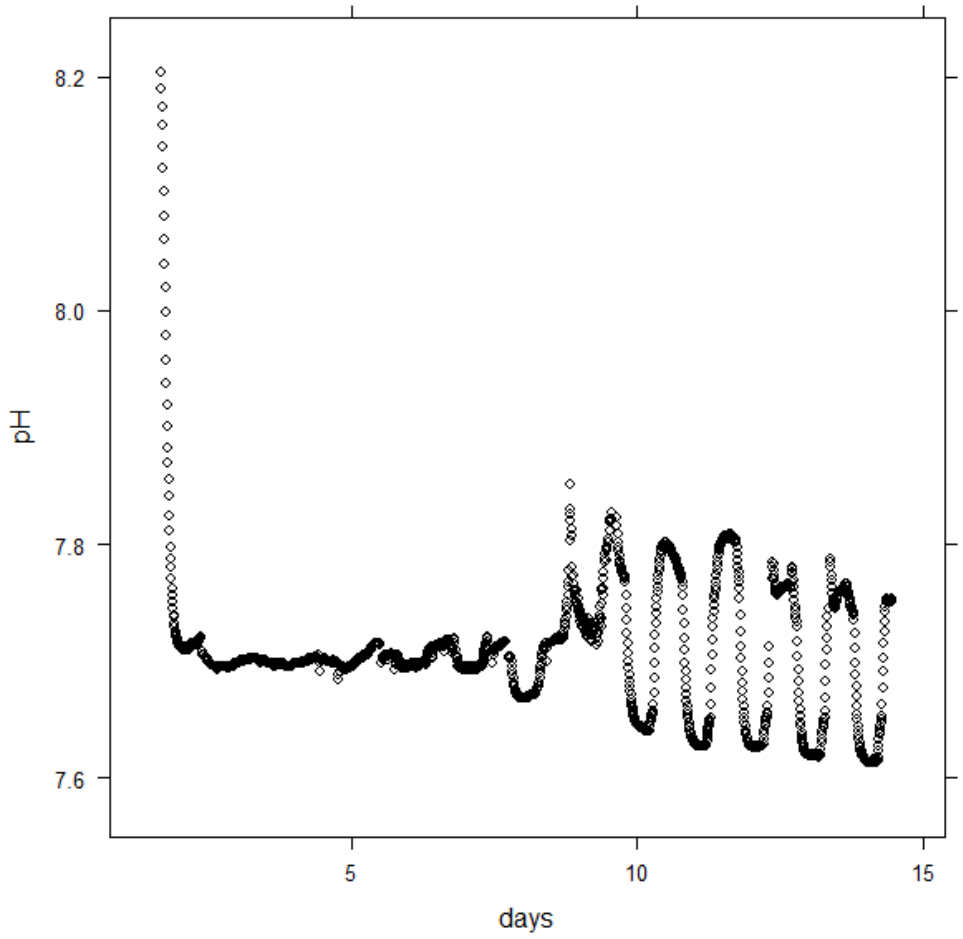


Figure 1 pH log of a *Synedropsis hyperborea* culture in full strength K medium, without the addition of tris buffer. 12/12hrs day / light / dark cycles start affecting the pH from day 7.

Figure 2 Pilot experiment flushing the headspace of a 750ml culture vessel on a shaker table with pre-mixed CO_2 air (top). A pH probe was inserted through the lid to log pH changes through the light-dark cycle (bottom).

While flushing the headspace with CO_2 – air mix circumvents the problems associated with bubbling, it only works well for small culture vessels with large surface area to volume ratios. The use of small culture vessels easily facilitates replication. However, any phytoplankton experiment that aims at

repeatedly sub-sampling a culture over time is ultimately limited by the volume of culture available. In order to avoid any disturbances to the culture physiology by removing excessive cell numbers, the total sample volume removed should be limited to no more than 10% of the culture volume (Feng *et al.* 2009). For our first phytoplankton experiments, described in Chapters 2 and 3, I therefore chose to use large culture vessels to allow repeated sampling of the phytoplankton cultures and CO_2 - bubbling to maintain stable CO_2 concentrations within the large culture volumes.

b. A semi – continuous phytoplankton culture system to simulate ocean acidification conditions

My first two experiments, described in Chapters 2 and 3, aimed to maintain phytoplankton in exponential growth over an extended period of time, in the order of several weeks. I chose a semi-continuous culture approach, which requires manual dilution on a regular basis to prevent the culture from exhausting all nutrients and entering a stationary phase. Phytoplankton cultures were maintained in exponential growth by dilution every two days with freshly prepared modified f/2 media that had been bubbled with CO_2 – air at target pCO_2. For each of the two experiments, twenty litre cultures of the diatom were grown in f/2 medium (Guillard and Ryther 1962; Guillard 1975) as inoculum for all four treatments. The inoculum was evenly transferred into four custom-made 45L transparent plastic growth bags (polyethylene, Entapack, Australia), setup in a refrigerator (Fig. 3) with temperatures averaging 3.5°C (range 2.4 - 4.2°C).

Phytoplankton samples were taken each week and replicate groups of krill larvae were fed regularly with the cultured phytoplankton. Therefore I used large culture volumes to allow repeated phytoplankton sampling for analyses and to conduct Antarctic larval krill feeding experiments from the same stock culture. The size of each culture bag, however, made replication impossible due to limited space for experiments.

Since the surface to volume ratio decreases with increasing volume, equilibration of the culture medium CO_2 concentration with a headspace is slow. I chose to gently bubble the cultures continuously with CO_2 - enriched air to maintain stable CO_2 concentrations in the culture. The control culture was bubbled with 0.2µm filtered ambient air. For the high CO_2 treatments, ambient 0.2µm filtered air was enriched with pure CO_2 gas (BOC, Australia) and then bubbled through each culture in the same way as the control. On the basis of an average atmospheric pCO_2 of 390ppm and set air flow rates, the required addition of CO_2 was calculated and regulated with mass flow controllers (Horiba STEC SEC-E-40). The air passed through silica gel to absorb moisture and reduce condensation in air lines. Details on the CO_2 system are described elsewhere (Kawaguchi *et al.* 2011).

Figure 3 Semi-continuous phytoplankton batch cultures in a temperature controlled refrigerator. CO_2-enriched air is continuously bubbled through the culture, after passing through silica gel, activated charcoal and a 0.2µm filter to remove any impurities.

c. Methodological considerations for Antarctic krill feeding experiments with high CO_2 - grown phytoplankton

The basic idea of the krill feeding experiments was to grow different phytoplankton species at elevated pCO_2, under the hypothesis that phytoplankton biochemistry might be altered by increased availability of CO_2 and decreased pH. Subsequently, these phytoplankton cultures were fed to krill larvae to determine any effects on growth, mortality and biochemistry. There are several concerns in such feeding experiments: adequate food supply, avoiding bacterial build-up, keeping the phytoplankton cells in suspension for effective filter feeding, maintaining target pH in the krill jars and minimizing exposure time of high CO_2-grown phytoplankton to ambient pCO_2 in the krill jars. Here I will describe the experimental approach undertaken for the first feeding experiments and how I addressed these issues.

Krill larvae were maintained in 5L plastic containers (Fig. 4) filled with 3L phytoplankton culture. To avoid starvation of krill and the resulting possible effects on krill biochemistry, fresh phytoplankton culture had to be provided regularly. Food supply to the larvae in this setup was not continuous and had to be renewed regularly by transferring the larvae manually from one container into a new one filled with fresh phytoplankton culture. The jars were not closed off to the environment and thus were not kept sterile. Bacteria could grow and build up on organic matter in the jar and on the krill and cause disease and / or interfere with the animal's ability to feed effectively. Regular water changes minimized bacterial build – up. However, preparing the new

culture solution and transferring the larvae was labour intensive and had the potential for damaging or losing animals.

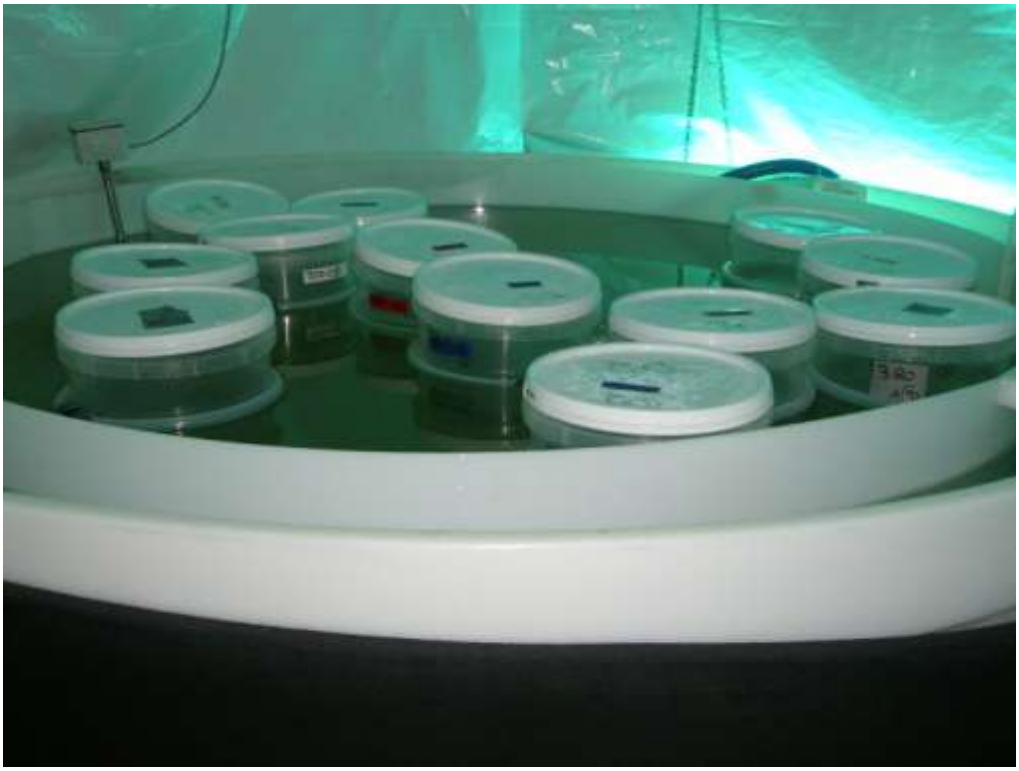


Figure 4 5L plastic container with krill larvae and 3L phytoplankton culture floating in 0.5°C krill holding tank.

To isolate the effect of altered phytoplankton biochemistry on krill from the effects of decreased seawater pH on krill, the pH of all high CO_2 grown phytoplankton cultures had to be adjusted to control pH before transferring krill into the culture. This, however, meant that high CO_2 grown phytoplankton cells were exposed to only ambient pCO_2 until they were eaten or the culture in the krill jar was replaced. It cannot be ruled out that the phytoplankton cells could respond quickly to the altered carbonate chemistry with changes to their biochemistry. Therefore the time between jar changes

and renewal of the culture had to be kept to a minimum. Due to time limitations water changes could only be performed every second day.

Food was present in surplus throughout those two days, as confirmed visually during krill transfers. Phytoplankton cells were resuspended manually during daily mortality.

II. Semi – continuous batch culture *versus* continuous culture system

a. Limitations of semi – continuous cultures

During the course of the semi – continuous culture experiments I identified a number of limitations and scope for improvements of the experimental setup and design. Here, I will discuss the limitations of a semi – continuous culture method and compare this approach with the continuous phytoplankton culture system described in Chapter 4.

Continuously bubbling CO_2 – enriched air through phytoplankton cultures did not maintain culture pCO_2 at the desired level during the first semi – continuous culture experiment (Chapter 2). This was likely due to the large volume of the culture vessels (~45L) compared to the volume of air delivered by bubbling. I addressed this issue in the second semi –continuous culture experiment by manually adjusting the volume of CO_2 added to air bubbling through the cultures. These adjustments were only made once per day, based on pH measurements and thus fluctuations between days were unavoidable.

Furthermore, to maintain exponential growth, cultures had to be diluted regularly. This was a labour intensive process and potentially changed cell physiology by altering nutrient concentrations in the medium during dilutions.

The semi – continuous culture system outlined in Chapters 2 and 3 was a good first step in conducting ocean acidification experiments with Antarctic phytoplankton. However, the system could not facilitate full replication of the phytoplankton treatments and deliver satisfactory stability of carbonate chemistry and nutrient concentrations without manual intervention. Thus, for the following experiments, the need to develop a culture system that could manage a vastly increased number of phytoplankton cultures, while reducing the labour intensity of maintaining exponential growth and target pCO_2 became apparent. The development of a continuous culture system was described in Chapter 4. In the following I will compare the advantages of continuous over semi – continuous culture system.

b. Comparison of semi – continuous and continuous culture approach - conclusions

Direct comparison of the stability of the carbonate chemistry between the semi – continuous and continuous cultures is compromised by the fact that different phytoplankton species, slightly different irradiance levels, light / dark cycles and different CO_2 concentrations were used. This is due to the fact that the primary goal of this research project was not to test different methods but to develop a reasonably cheap, flexible and reliable system to conduct ocean acidification experiments with Antarctic phytoplankton. However, with the above limitation in mind, it is still possible to qualitatively compare the performance of semi – continuous and continuous culture method.

Carbonate chemistry

In ocean acidification experiments it is vital to avoid overlap of experimental pCO_2 treatments in order to detect significant changes between treatments. Standard deviations in pH and calculated pCO_2 were smaller using a continuous system than when using a semi – continuous culture system (Table 1). This improves the ability to detect CO_2 – induced effects in phytoplankton cultures. The average range in pH values within treatments was close to three times larger in the semi – continuous culture of *Synedropsis hyperborea* compared to the continuous cultures. Furthermore, the calculated CO_2 concentrations were closer to our target values in the continuous system compared to the semi – continuous cultures (Table 1).

Table 1 Measured pH and calculated pCO_2 of semi - continuous and continuous cultures. Headings are target pCO_2 (µatm). SD = standard deviation, range = difference between highest and lowest recorded pH, measured pCO_2 values were calculated using CO2SYS from alkalinity and pH, positive (negative) values mean the actual CO_2 concentration was higher (lower) than the target value.

Semi – continuous cultures

Synedropsis hyperborea

Target pCO_2 [µatm]	**390**	**500**	**750**	**950**
Average pH ± SD	8.37 ± 0.10	8.18 ± 0.11	7.94 ± 0.06	7.82 ± 0.04
pH range	0.53	0.65	0.35	0.23
Calculated pCO_2 ± SD	159 ± 61	263 ± 88	527 ± 92	727 ± 83
Diff from target	- 231	- 237	- 223	- 223

Pseudo-nitzschia subcurvata

Target pCO_2 [µatm]	**390**	**500**	**750**	**950**
Average pH ± SD	8.15 ± 0.05	8.01 ± 0.05	7.82 ± 0.04	7.72 ± 0.04
pH range	0.18	0.25	0.16	0.16
Calculated pCO2 ± SD	326 ± 27	431 ± 62	712 ± 62	896 ± 68
Diff from target	- 64	- 70	- 38	- 54

Continuous cultures

Fragilariopsis cylindrus

Target pCO_2 [µatm]	**390**	**500**	**750**	**950**
Average pH ± SD	8.02 ± 0.03	7.88 ± 0.03	7.77 ± 0.04	7.69 ± 0.03
pH range	0.13	0.15	0.20	0.17
Calculated *p*CO2 ± SD	428 ± 34	590 ± 46	771 ± 67	950 ± 78
Diff from target	+ 38	+ 20	+ 21	± 0

Gymnodinium sp.

Target pCO_2 [µatm]	**390**	**500**	**750**	**950**
Average pH ± SD	8.02 ± 0.04	7.89 ± 0.03	7.75 ± 0.04	7.70 ± 0.03
pH range	0.21	0.21	0.21	0.21
Calculated pCO_2 ± SD	458 ± 48	580 ± 49	797 ± 79	973 ± 82
Diff from target	+ 68	+ 10	+ 47	+ 23

Pyramimonas gelidicola

Target pCO_2 [µatm]	**390**	**500**	**750**	**950**
Average pH ± SD	8.04 ± 0.04	7.87 ± 0.03	7.75 ± 0.04	7.67 ± 0.04
pH range	0.17	0.13	0.16	0.17
Calculated pCO_2 ± SD	400 ± 41	612 ± 54	806 ± 80	977 ± 106
Diff from target	+ 10	+ 42	+ 56	+ 27

Phaeocystis antarctica

Target pCO_2 [µatm]	**390**	**500**	**750**	**950**
Average pH ± SD	8.02 ± 0.03	7.86 ± 0.04	7.76 ± 0.04	7.67 ± 0.03
pH range	0.14	0.15	0.19	0.14
Calculated pCO_2 ± SD	413 ± 31	644 ± 62	805 ± 76	993 ± 83
Diff from target	+ 23	+ 74	+ 55	+ 43

Cost effectiveness and practicality

The initial cost for the multi – channel peristaltic pumps made the continuous system more expensive than a semi – continuous system. However, labour was greatly reduced in the automated continuous culture setup since culture dilution was conducted by the peristaltic pumps and culture medium CO_2 did not require manual adjustments. As manual maintenance of the individual culture vessels was minimal, replication of treatments could be increased compared to the semi – continuous culture experiments. This vastly improved statistical power and the ability to detect significant differences between treatments as variations between culture vessels and actual treatment effects could be distinguished.

Conclusion

Our continuous culture system was a good first step, but some improvements could be made to this system. These include single – channel pumps for each culture vessel for better accuracy in medium delivery and / or flexibility in adjusting the in – flow of medium individually for each culture vessel. The biggest challenge for the continuous method, however, is maintaining the closed system sterile. Assuring 100% sterility of all components of such a fairly complex system at the assembly point is a challenge. Culture vessels, teflon and silicon fittings can be autoclaved or purchased sterile; air and culture medium can be filter sterilized. Yet, a system as described here, involves a great length of tubing to deliver medium and / or culture that cannot be autoclaved and thus the only practical way to achieve sterility is to flush the tubing with 70 – 80% ethanol and then rinse with deionised and UV-

treated water or filter sterilized seawater. The conditions for bacterial growth are ideal with constant flow of nutrients through the system, so microbial contamination at any stage in the assembly process must be avoided. Flushing the whole system with a mild bactericide, such as sodium azide, and a subsequent rinse with either deionised and UV treated water or filter sterilized seawater once the system is fully assembled could help minimize the danger of bacterial contamination.

In conclusion, our continuous culture system was a vast improvement over previously employed semi – continuous culture methodologies and the flexibility of this newly designed system made it adaptable to incorporate more complex experimental designs, such as described in the following chapter.

III. Development of a flow – through system for krill feeding experiments under ocean acidification – combining stressors

a. Combining direct and indirect effects of ocean acidification on Antarctic krill

Antarctic krill could be susceptible to direct and indirect impacts of ocean acidification. Formation of the calcium carbonate layer that supports the chitinous membrane of krill could be negatively affected by decreased concentrations of CO_3^{2-} and lower pH. Metabolic rate, growth and reproduction could be suppressed by the increase in seawater H^+ concentration (Whiteley 2011). A deterioration of phytoplankton nutritional quality can increase krill larval mortality rate as seen in Chapter 3. The first two experiments with krill larvae investigated the effect of altered food quality on krill by only subjecting the phytoplankton cultures to elevated pCO_2 and not the larvae. Using this approach allowed the effect of altered food quality to be isolated from the direct effect of ocean acidification on krill. However, in nature direct and indirect effects of ocean acidification will occur in combination and therefore the goal of the final experiment was to determine the combined effects of elevated pCO_2 on krill. To achieve this goal I had to further develop the existing experimental facilities.

I will therefore briefly summarize the limitations of previous phytoplankton and krill experiments and then describe the development of a flow – through

system for the final krill feeding experiment with the diatom *Pseudo-nitzschia subcurvata*.

b. Experimental design and methodology

Experimental design

I wanted to retain the ability to distinguish between the impact and proportional contribution of the separate stressors, in this case, low pH and high CO_2 and altered nutritional quality of the phytoplankton. For that reason the experimental design needed to include:

- a control treatment, where krill and phytoplankton were exposed to ambient pCO_2 (low low, LL),
- a high pCO_2 treatment, where both krill and phytoplankton were exposed to elevated CO_2 concentration (high high, HH),
- the cross – over treatments, i.e. krill exposed to elevated pCO_2 seawater feeding on phytoplankton grown at ambient CO_2 levels (high low, HL) and krill exposed to ambient pCO_2 seawater feeding on phytoplankton grown at elevated pCO_2 (low high, LH).

Methodology

Out of the four treatments, LL, HH, HL and LH, the latter two cross – over treatments are logistically most challenging. This is due to the fact that the CO_2 concentration of the phytoplankton culture had to be altered and maintained before krill was exposed to it.

There are two approaches for this kind of experimental design:

- Semi – continuous approach: manual adjustment of phytoplankton culture to target pCO_2 in the experimental vessel and feeding of krill at discrete time points
- Continuous approach: continuously provide phytoplankton into the krill vessels, which are continuously maintained at target pCO_2

Semi – continuous approach

During the first two krill feeding experiments (Chapters 2 and 3) the pCO_2 and pH of phytoplankton cultures that had been grown at elevated pCO_2 were adjusted to ambient levels before transferring the krill larvae into the cultures. However, using this approach meant that phytoplankton cells grown at elevated pCO_2 were exposed to ambient CO_2 levels for two days while krill larvae were feeding on it. It was logistically impossible to reduce the time that phytoplankton was exposed to altered carbonate chemistry in the krill jars, since phytoplankton cultures had to be adjusted manually before krill larvae could be transferred individually into these freshly prepared cultures. This was a very labour intensive process and could only be completed every two days. As the study by Rossoll *et al.* (2012) showed, it is possible that the biochemistry of phytoplankton changes within hours of exposure to altered carbonate chemistry. The results of the 48h experiment described in Chapter 6, however, suggest that the sudden change from high pCO_2 environment in culture bag to ambient pCO_2 in the krill jar likely resulted in less change in *Pseudo-nitzschia subcurvata* biochemistry than the gradual change in nutrient concentration over the two days, with such changes further compounded by the addition of nutrients from krill faeces.

Continuous approach

To address the issues identified from the semi – continuous setup, the continuous setup of the phytoplankton experiment was extended to incorporate the krill feeding experiment. Instead of collecting phytoplankton culture in glass bottles, the overflow was pumped into individual krill jars by a second peristaltic pump. This reduced the time phytoplankton was exposed to a carbonate chemistry different from the culture vessel it came from, and food was supplied to krill constantly rather than every two days only. One peristaltic pump continuously supplied the cultures with fresh medium, while the other peristaltic pump removed an equivalent volume of culture from the culture bags and pumped it into the respective krill jars. Krill larvae were maintained in custom – made plastic containers that allowed food to flow through without losing krill larvae. Two 700mm^2 windows were cut out on opposite sides of a 2L plastic container and a 80µm mesh glued over. Three holes were drilled into the screw - on lid, one for the food line, one for the air line and one to release pressure. This plastic container was placed into a custom – made acrylic jar of 15cm diameter and 18.3cm height (Fig. 5).

The food line dripped phytoplankton culture continuously into the inner jar and excess liquid overflowed out of the acrylic outer jar into the surrounding tank. Use of the outer jars prevented reverse flow of surrounding seawater back into the krill jar. Silicone tubing was used as a spacer around some of the jars to prevent cross contamination between treatments (Fig. 5.).

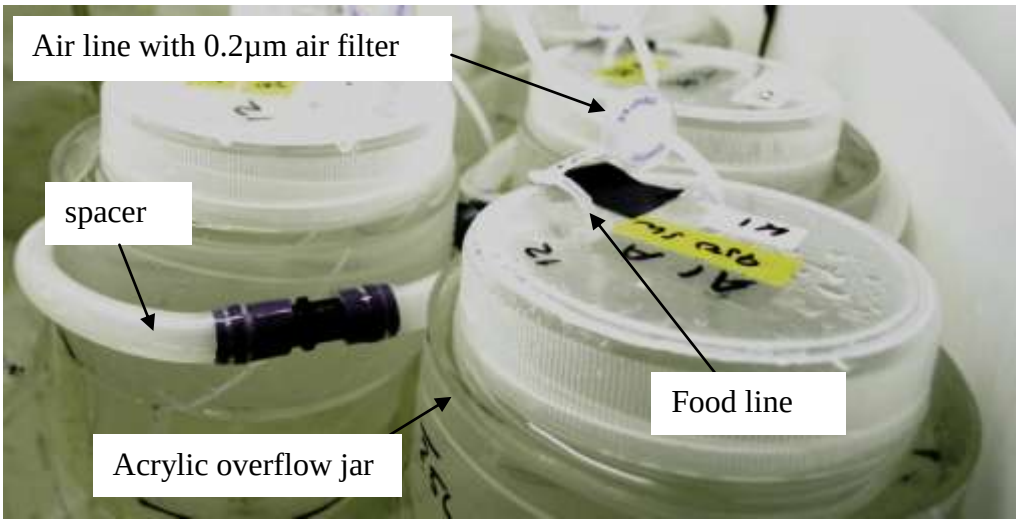


Figure 5 Continuous krill feeding setup. Plastic jars with mesh windows were placed into acrylic overflow jars. Phytoplankton culture was continuously pumped into the inner plastic jar through the food line. Excess culture overflowed out of the acrylic overflow jar into the surrounding tank. CO_2 concentrations were maintained by gently bubbling with 0.2µm – filtered air or CO_2 – enriched air through the air line.

The pH in the krill jars was maintained irrespective of the culture pH by gently bubbling the jars with either ambient air or 950ppm CO_2 – air mixes. CO_2 was added to ambient air in the same way as described in Chapter 2 and 3. Two manifolds split the main air lines into twelve (2x6) lines to supply the jars (Fig. 6). Each line could be turned on and off and bubbling intensity adjusted individually with clamps. Sterility was achieved by use of 0.2µm in – line filters. Teflon was used downstream from the air filter because it can be autoclaved for sterility and the small diameter produced small bubbles to minimize turbulence in the krill jars. Bubbling the jars had the additional benefit of keeping the phytoplankton cells in suspension for the filter feeding krill larvae, in contrast to the static approach in the semi – continuous feeding method outlined in Chapter 3. The krill jars were arranged on a transparent acrylic shelf, suspended from the ceiling. The depth to which the jars were submerged in the 0.5°C chilled krill holding tank could be adjusted so that the

top of the jars were just above the main water level while keeping the jars cool (Fig 6).

Use of the continuous system for the final krill feeding experiment also addressed the lack of replication during previous experiments, which meant that the results of biochemical changes in the semi – continuous phytoplankton cultures could not unequivocally be attributed to either changed CO_2 concentrations or vessel to vessel variation. The new experimental design could accommodate 12 phytoplankton cultures, with three vessels used per treatment.

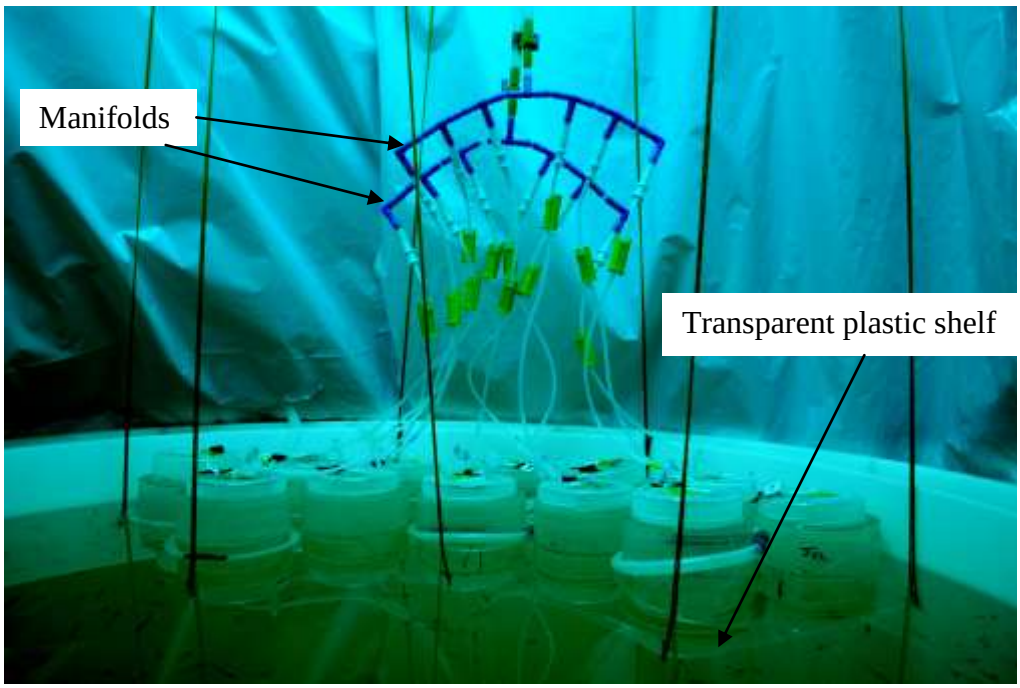


Figure 6 Krill jars on a transparent plastic shelf in a 0.5°C chilled krill holding tank. Ambient air and 950ppm CO_2-air mix was bubbled into the krill jars to maintain stable pH conditions.

Sampling regime

The sampling regime for the final experiment was generally the same as for previous krill feeding experiments. Once the phytoplankton cultures had acclimated, culture was delivered to the krill jars. Phytoplankton cultures in the culture bags as well as in the krill jars were sampled for C:N ratio, fatty acid and pigment content and composition and cellular carbohydrate concentration on a weekly basis. To complement the pigment data and monitor photosynthetic efficiency, measurements with a Pulse – Amplitude – Modulation fluorometer were carried out. Daily mortality checks and collection of krill moults were the main krill response parameter together with the analysis of liquid nitrogen frozen krill, for C:N ratio and fatty acid content and composition, after a time period comparative to the second krill feeding experiment (~ four weeks).

c. Identification of problems during experimental operation

Several days after the start of pumping phytoplankton culture into the krill, cell density in the phytoplankton culture bags declined rapidly. The most likely cause for the rapid decline in phytoplankton cell densities was the toxic material from the Tygon tubing (Masterflex) within the peristaltic pump delivering media to the culture bags. The majority of this tubing running over the pump head of the peristaltic pump had worn over time and ruptured, and the damaged tubing had to be replaced as soon as the problem was identified. Prior to replacing the torn lines the new tubing was not washed other than with a few millilitres of 80% ethanol for sterilisation since this was an ad hoc

solution to an unexpected event. The decline in cell densities coincided with the date of replacing the tubing. A literature search revealed that unwashed Tygon tubing can be toxic to phytoplankton (Price *et al.* 1986). Before our experiment was started, the peristaltic pumps were tested and calibrated extensively, which meant that the tubing was washed with hundreds of litres of sterile deionised water and culture medium before delivering media to the phytoplankton cultures. According to Price *et al.* (1986), washing a 1m Tygon piece of 0.6cm diameter with as little as 200ml seawater is enough to remove the toxic effect.

d. Discussion: flow – through system *versus* semi – continuous culturing for krill feeding experiments

The continuous feeding experiment described above and the semi – continuous experiment described in Chapters 2 and 3 used different approaches and thus had different strength and shortcomings which will be discussed below.

Delivering phytoplankton to the krill

During the semi – continuous experiments it was deemed sufficient to provide the larvae with fresh food only every second day, since food was always in surplus, as confirmed visually during the daily checks, and due to time limitations in preparing fresh jars on a daily basis. However, a change in phytoplankton biochemistry, when cells were grown under elevated pCO_2 and then transferred into control seawater pH and pCO_2, could not be ruled out. Rossoll *et al.* (2012) found that *Thalassiosira pseudonana* cells growing under

elevated pCO_2 changed their fatty acid profile within hours of being transferred from elevated pCO_2 to ambient pCO_2 seawater. The 48h experiment described in Chapter 6 also suggests that a difference in nutrient concentration between the culture bag and the krill jar could affect phytoplankton biochemistry. During the flow – through system food supply was continuous, thereby minimizing the risk of food limitation in between checks and sampling, and phytoplankton cells were replaced continuously to minimize the resident time of the cells in altered pCO_2 and nutrient concentration.

Keeping phytoplankton cells in suspension for filter feeding larvae

Although the containers were in constant gentle motion while floating in the big holding tank during the semi – continuous setup, settling of phytoplankton cells in between daily checks could not be avoided. Some form of constant mixing inside the containers would be advantageous as settled phytoplankton reduce the availability of cells for the filter feeding larvae. Gentle bubbling of the krill jars during the continuous feeding experiment provided constant agitation and reduced the settling of phytoplankton cells to the bottom of the jar.

Combining direct and indirect ocean acidification impacts

The semi – continuous feeding experiments isolated the effects of altered phytoplankton biochemistry on krill from the possible effects of reduced seawater pH on krill. This approach is helpful in identifying individual drivers of change, however naturally, phytoplankton and krill will be exposed to the

same pCO_2 environment with possible synergistic effects. The setup of the continuous feeding experiment provided the opportunity to do both, i.e. isolate the effects of phytoplankton grown under elevated pCO_2 on krill and the combination of the effects of reduced pH and altered food quality on krill. To retain the resolution of each factor, krill was exposed to high CO_2 grown phytoplankton in high CO_2 seawater, as well as high CO_2 phytoplankton in ambient seawater and phytoplankton grown under ambient seawater in high CO_2 seawater for a full factorial design.

Labour intensity

Transferring krill larvae into fresh jars every second day during the semi – continuous system was laborious and increased handling stress and risk of damaging or losing animals. The benefit, however, lay in the regular removal of bacterial build – up on organic and faecal matter and the ability to regularly control the phytoplankton cell concentration in the krill jars across treatments regardless of phytoplankton growth rates.

e. Conclusion

In conclusion the new flow - through system was a vast improvement over previous experiments and worked well until the decline in phytoplankton cultures, which was most likely caused by the toxic effect of the new Tygon tubing. The continuous feeding setup of the flow - through system was less labour intensive and meant less handling stress for the animals. Due to continuous bubbling with CO_2 – air, phytoplankton cells were kept in suspension. This opened up the possibility to include cross – over treatments

where the pCO_2 concentrations that the phytoplankton were grown in were different to that at which krill were maintained. However, the failure of this experiment occurred due to unforeseen circumstances and provided an important reminder of the challenges faced when setting up laboratory experiments. This experiment could not be repeated due to time constraints but provided useful experience and results for future experiments.

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